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(54) Title: HUMAN POLYPEPTIDES CAUSING OR LEADING TO THE KILLING OF CELLS INCLUDING LYMPHOID TUMOR CELLS

(57) Abstract: The present invention relates to polypeptide compositions which bind to cell surface epitopes and, in multivalent forms, cause or lead to the killing of cells including lymphoid tumor cells, and in the case of monovalent forms, cause immunosuppression or otherwise inhibit activation of lymphocytes. The invention further relates to nucleic acids encoding the polypeptides, methods for the production of the polypeptides, methods for killing cells, methods for immunosuppressing a patient, pharmaceutical, diagnostic and multivalent compositions and kits comprising the polypeptides and uses of the polypeptides.

**Human polypeptides causing or leading to the killing of cells including
lymphoid tumor cells**

Background of the Invention

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Every mammalian species, which has been studied to date, carries a cluster of genes coding for the so-called major histocompatibility complex (MHC). This tightly linked cluster of genes code for surface antigens, which play a central role in the development of both humoral and cell-mediated immune responses. In humans the 10 products coded for by the MHC are referred to as Human Leukocyte Antigens or HLA. The MHC-genes are organized into regions encoding three classes of molecules, class I to III.

Class I MHC molecules are 45 kD transmembrane glycoproteins, noncovalently 15 associated with another glycoprotein, the 12 kD beta-2 microglobulin (Brown et al., 1993). The latter is not inserted into the cell membrane, and is encoded outside the MHC. Human class I molecules are of three different isotypes, termed HLA-A, -B, and -C, encoded in separate loci. The tissue expression of class I molecules is ubiquitous and codominant. MHC class I molecules present peptide antigens necessary for the 20 activation of cytotoxic T-cells.

Class II MHC molecules are noncovalently associated heterodimers of two transmembrane glycoproteins, the 35 kD α chain and the 28 kD β chain (Brown et al., 1993). In humans, class II molecules occur as three different isotypes, termed human 25 leukocyte antigen DR (HLA-DR), HLA-DP and HLA-DQ. Polymorphism in DR is restricted to the β chain, whereas both chains are polymorphic in the DP and DQ isotypes. Class II molecules are expressed codominantly, but in contrast to class I, exhibit a restricted tissue distribution: they are present only on the surface of cells of the immune system, for example dendritic cells, macrophages, B lymphocytes, and 30 activated T lymphocytes. They are also expressed on human adrenocortical cells in the zona reticularis of normal adrenal glands and on granulosa-lutein cells in corpora lutea of normal ovaries (Kahoury et al., 1990). Their major biological role is to bind antigenic peptides and present them on the surface of antigen presenting cells (APC) for recognition by CD4 helper T (Th) lymphocytes (Babbitt et al., 1985). MHC class II

molecules can also be expressed on the surface of non-immune system cells, for example, cells that express MHC class II molecules during a pathological inflammatory response. These cells may include synovial cells, endothelial cells, thyroid stromal cells and glial cells.

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Class III MHC molecules are also associated with immune responses, but encode somewhat different products. These include a number of soluble serum proteins, enzymes and proteins like tumor necrosis factor or steroid 21-hydroxylase enzymes. In humans, class III molecules occur as three different isotypes, termed Ca, C2 and

10 Bf (Kuby, 1994).

Since Th cell activation is a crucial event of the initiation of virtually all immune responses and is mediated through class II molecules, class II MHC offers itself as a target for immunomodulation (Baxevanis et al., 1980; Rosenbaum et al., 1981;

15 Adorini et al., 1988). Besides peptide presentation, class II molecules can transduce various signals that influence the physiology of APC. Such signals arise by the interaction of multiple class II molecules with an antibody or with the antigen receptor of Th cells (Vidovic et al., 1995a; Vidovic et al., 1995b), and can induce B cell activation and immunoglobulin secretion (Cambier et al., 1991; Palacios et al., 1983),
20 cytokine production by monocytes (Palacios, 1985) as well as the up-regulation of co-stimulatory (Nabavi et al., 1992) and cell adhesion molecules (Mourad et al., 1990).

There is also a set of observations suggesting that class II ligation, under certain conditions, can lead to cell growth arrest or be cytotoxic. Ligation under these

25 conditions is the interaction of a polypeptide with a class II MHC molecule. There is substantial contradiction about the latter effects and their possible mechanisms. Certain authors claim that formation of a complex of class II molecules on B cells leads to growth inhibition (Vaickus et al., 1989; Kabelitz et al., 1989), whereas according to others class II complex formation results in cell death (Vidovic et al.,
30 1995a; Newell et al., 1993; Truman et al., 1994; Truman et al., 1997; Drenou et al., 1999). In certain experimental systems, the phenomenon was observed with resting B cells only (Newell et al., 1993), or in other systems with activated B cells only (Vidovic et al., 1995a; Truman et al., 1994).

Based on these observations, anti-class II monoclonal antibodies (mAbs) have been envisaged for a number of years as therapeutic candidates. Indeed, this proposal has been supported by the beneficial effect of mouse-derived anti-class II mAbs in a series of animal disease models (Waldor et al., 1983; Jonker et al., 1988; Stevens et 5 al., 1990; Smith et al., 1994; Vidovic & Torral, 1998; Vidovic & Laus, 2000).

Despite these early supporting data, to date no anti-MHC class II mAb of human composition has been described that displays the desired cytotoxic and other biological properties which may include affinity, efficiency of killing and selectivity. 10 Indeed, despite the relative ease by which mouse-derived mAbs may be derived, work using mouse-derived mAbs has demonstrated the difficulty of obtaining an antibody with the desired biological properties. For example, significant and not fully understood differences were observed in the T cell inhibitory capacity of different murine anti-class II mAbs (Naquet et al., 1983). Furthermore, the application of 15 certain mouse-derived mAbs *in vivo* was associated with unexpected side effects, sometimes resulting in death of laboratory primates (Billing et al., 1983; Jonker et al., 1991).

It is generally accepted that mouse-derived mAbs (including chimeric and so-called 20 'humanized' mAbs) carry an increased risk of generating an adverse immune response (Human anti-murine antibody – HAMA) in patients compared to treatment with a human mAb (for example, Vose et al, 2000; Kashmiri et al., 2001). This risk is potentially increased when treating chronic diseases such as rheumatoid arthritis or multiple sclerosis with any mouse-derived mAb or where regular treatment may be 25 required, for example in the treatment of certain cancers; prolonged exposure of the human immune system to a non-human molecule often leads to the development of an adverse immune reaction. Furthermore, it has proven very difficult to obtain mouse-derived antibodies with the desired specificity or affinity to the desired antigen (Pichla et al. 1997). Such observation may significantly reduce the overall therapeutic 30 effect or advantage provided by mouse-derived mAbs. Examples of disadvantages for mouse-derived mAbs may include the following. First, mouse-derived mAbs may be limited in the medical conditions or length of treatment for a condition for which they are appropriate. Second, the dose rate for mouse-derived mAbs may need to be relatively high in order to compensate for a relatively low affinity or therapeutic effect,

hence making the dose not only more severe but potentially more immunogenic and perhaps dangerous. Third, such restrictions in suitable treatment regimes and high-dose rates requiring high production amounts may significantly add to the cost of treatment and could mean that such a mouse-derived mAb be uneconomical to
5 develop as a commercial therapeutic. Finally, even if a mouse mAb could be identified that displayed the desired specificity or affinity, often these desired features are detrimentally affected during the 'humanization' or 'chimerization' procedures necessary to reduce immunogenic potential (Slavin-Chiorini et al., 1997). Once a mouse-derived mAb has been 'humanized' or chimerized, then it is very difficult to
10 optimize its specificity or affinity.

The art has sought over a number of years for anti-MHC class II mAbs of human composition that show biological properties suitable for use in a pharmaceutical composition for the treatment of humans. Workers in the field have practiced the
15 process steps of first identifying a mouse-derived mAb, and then modifying the structure of this mAb with the aim of improving immunotolerance of this non-human molecule for human patients (for further details, see Jones et al., 1986; Riechmann et al., 1988; Presta, 1992). This modification is typically made using so-called 'humanization' procedures or by fabricating a human-mouse chimeric mAb. Other
20 workers have attempted to identify human antibodies that bind to human antigens having desired properties within natural repertoires of human antibody diversity. For example, by exploring the foetal-tolerance mechanism in pregnant women (Bonagura et al., 1987) or by panning libraries of natural diversities of antibodies (Stausbøl-Grøn et al., 1996; Winter et al., 1994). However, to date no anti-MHC class II mAb of
25 human composition has been described that displays the desired biological properties of cytotoxicity, selectivity, specificity, low immunogenicity and affinity.

For therapeutic purposes a polypeptide reacting with many or at least most of the common allelic forms of a human class II MHC molecule would be desirable – e.g., to
30 enable its use in diverse patient populations. Moreover, the candidate polypeptide should be cytotoxic to a wide range of lymphoid tumors, and preferably is cytotoxic by way of a mechanism common to such a range of tumor cells. To allow for a wide range of possible applications, the polypeptide desired should mediate its cytotoxic effect without the dependence on further components of the immune system. For

therapeutic purposes most patients receive for the treatment of e.g. cancer standard chemo- or radiotherapy. Most of these treatments leave the patient immunocompromised. Any additional treatment that relies on an intact immune system is therefore likely to fail. The underlying problem is further demonstrated in
5 humans who suffer from a disease that destroys the immune system, e.g. HIV. Opportunistic infections and malignant transformations are able to escape the immune-surveillance and cause further complications.

Summary of the Invention

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One aspect of the present invention relates to a composition including a polypeptide comprising at least one antibody-based antigen-binding domain of human composition with binding specificity for an antigen expressed on the surface of a human cell, wherein treating cells expressing the antigen with a multivalent polypeptide having two or more of
15 said antigen binding domains causes or leads to killing of the cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain in preferred embodiments the antigen is an MHC antigen, preferably an MHC class II antigen, such as DR/DP/DQ or DR. For instance, in certain preferred embodiments, the subject compositions include a polypeptide comprising at least one
20 antibody-based antigen-binding domain which binds to human HLA DR with a K_d of 1 μ M, 100nM, 10nM or even 1nM or less.

25

Another aspect of the present invention provides a composition including a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition with binding specificity for human HLA DR. Treating cells expressing HLA DR with the multivalent polypeptide causes or leads to killing of the cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain preferred embodiments, the said antigen-binding domains individually bind to the human HLA DR with a K_d of 1 μ M, 100nM, 10nM or even 1nM or less. In certain preferred
30 embodiments, the multivalent polypeptide has an EC₅₀ of 100 nM, 10nM or even 1nM or less for killing activated lymphoid cells, transformed cells and/or lymphoid tumor cells.

35

Still another aspect of the present invention provides a composition including a polypeptide comprising at least one antibody-based antigen-binding domain that binds to human HLA DR with a K_d of 1 μ M, 100nM, 10nM or even 1nM or less, the antigen-binding

domain being isolated by a method which includes isolation of human VL and VH domains from a recombinant antibody library by ability to bind to at least one epitope of human HLA DR. Treating a cell expressing HLA DR with a multivalent polypeptide having two or more of the antigen binding domains causes or leads to killing of the cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain embodiments, the method for isolating the antigen-binding domain includes the further steps of:

- 5 a. generating a library of variants of at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
- 10 b. isolation of VL and VH domains from the library of variants by ability to bind to human HLA DR with a K_d of $1\mu M$ or less.

In certain preferred embodiments, the composition of the present invention can be characterized as including multivalent polypeptides having an EC_{50} for killing transformed 15 cells at least 5-fold lower than the EC_{50} for killing normal cells, and even more preferably at least 10-fold, 100-fold and even 1000-fold less than for killing normal cells.

In certain preferred embodiments, the composition of the present invention are characterized as including multivalent polypeptides having an EC_{50} for killing activated 20 cells at least 5-fold lower than the EC_{50} for killing unactivated cells, and even more preferably at least 10-fold, 100-fold and even 1000-fold less than for killing unactivated cells.

In certain preferred embodiments, the composition of the present invention are characterized as including multivalent polypeptides having an EC_{50} of $50nM$ or less for killing transformed cells, and even more preferably an EC_{50} of less than $10nM$, $1nM$ and even $0.1nM$. In certain embodiments, the subject multivalent polypeptides have an EC_{50} for killing activated lymphoid cells, transformed cells and/or lymphoid tumor cells of 25 $100nM$, $10nM$ or even $1nM$ or less.

30 In certain embodiments, the subject compositions including multivalent polypeptides selectively kill activated lymphoid cells. For example, such multivalent forms of the subject compositions can be used to kill activated lymphoid cells are lymphoid tumor cells representing a disease selected from B cell non-Hodgkin lymphoma, B cell 35 lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma,

chronic myeloid leukemia, chronic lymphoid leukemia, and multiple myeloid leukemia. Exemplary activated lymphoid tumor cells which can be killed include Priess, GRANTA-519, KARPAS-422, KARPAS-299, DOHH-2, SR-786, MHH-CALL-4, MN-60, BJAB, RAJI, L-428, HDLM-2, HD-MY-Z, KM-H2, L1236, BONNA-12, HC-1, NALM-1, L-363, EOL-1, 5 LP-1, RPMI-8226, and MHH-PREB-1 cell lines. In certain preferred embodiments, the subject compositions have an EC₅₀ of 100nM or less, and preferably less than 10nM or even 1nM, for killing at least one of B cell lymphoma cells and T cell lymphoma cells selected from the list of KARPAS-422, DOHH-2, SR-7, MHH-CALL-4, MN-60, HD-MY-Z, NALM-1 and LP-1. In certain instances, to effect cell killing, the target cells may require 10 further activation or pre-activation, such as by incubation with Lipopolysaccharide (LPS, 10 µg/ml), Interferon-gamma (IFN-γ, Roche, 40 ng/ml) and/or phyto-hemagglutinin (PHA, 5 µg/ml) to name but a few.

In certain embodiments, the multivalent forms of the subject compositions can be used to 15 kill non-lymphoid cells that express MHC class II molecules.

Certain embodiments, one or more the antigen binding domains of the subject compositions bind to the β-chain of HLA-DR, e.g., the antigen-binding domain binds to the first domain of the β-chain of HLA-DR.
20 In certain other embodiments, one or more the antigen binding domains of the subject compositions bind to the α-chain of HLA-DR, e.g., the antigen-binding domain binds to the first domain of the α-chain of HLA-DR.

25 In certain preferred embodiments, the the antigen binding domain(s) of the subject compositions bind to one or more HLA-DR types selected from the group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402, DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRW53-B4*0101 and DRW52-B3*0101. In preferred embodiments, the the antigen binding domains of the subject compositions 30 provide broad-DR reactivity, that is, the antigen-binding domain(s) of a given composition binds to epitopes on at least 5 different of said HLA-DR types. In certain embodiments, the antigen binding domain(s) of a polypeptide(s) of the subject compositions binds to a plurality of HLA-DR types as to bind to HLA DR expressing cells for at least 60 percent of the human population, more preferably at least 75 percent, and even more preferably 85 35 percent of the human population.

- In certain embodiments, the antigen-binding domains of the subject compositions include a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10,
5 MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.
- 10 In certain embodiments, the antigen-binding domains of the subject compositions include a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3, VL CDR1 And VL CDR3 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

In a further preferred embodiment, the antigen-binding domain is modified compared to a parental antigen-binding domain of the present invention by addition, deletion and/or substitution of amino acid residues, while maintaining the properties according to the present invention, or improving one or more of said properties, of said parental antigen-binding domain. This may include, but is not limited to, the modification of a nucleic acid sequence encoding a parental antigen-binding domain for cloning purposes, the modification of CDR regions in order to improve or modify antigen-binding affinity and/or specificity, including the exchange of one or more CDR sequences of a parental antigen-binding domain by corresponding CDR sequences from one or more different antigen-binding domains, and the addition of peptide sequences for detection and/or purification purposes. It is well within the scope of one of ordinary skill in the art to identify positions in a given parental antigen-binding domain where an addition, deletion and/or substitution should occur, to design and pursue the approach to achieve said addition, deletion and/or substitution, and to test or assay whether the modified antigen-binding domain has maintained the properties of, or exhibits one or more improved properties compared to, the parental antigen-binding domain. Furthermore, one of ordinary skill would be able to design approaches where collections or libraries of modified antigen-binding domains are designed, constructed and screened to identify one or more modified antigen-binding domain which have maintained the properties, or exhibit one or

more improved properties compared to the parental antigen-binding domain. In one example, the first amino acid residue of a HuCAL VH domain comprised in any antigen-binding domain or the present invention, which is either E or Q depending on the expression construct, may be exchanged by Q or E, respectively. Preferred regions to optimize an antigen-binding domain by designing, constructing and screening collections or libraries of modified antigen-binding domains according to the present invention comprise the CDR regions, and most preferably CDR3 of VH and VL, CDR1 of VL and CDR2 of VH domains.

10 In certain embodiments, the antigen-binding domains includes a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and/or

15 wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

wherein each n independently represents any amino acid residue. For instance, the VH CDR3 sequence can be SPRYGAFDY and/or the VL CDR3 sequence can be QSYDLIRH or QSYDMNVH.

20

In certain embodiments, the antigen-binding domains of the subject antigen-binding domain competes for antigen binding with an antibody including a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

25

nnnnRGnFDn

each n independently represents any amino acid residue; and/or

the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

each n independently represents any amino acid residue. For instance, the VH CDR3

30 sequence can be SPRYGAFDY and/or the VL CDR3 sequence can be QSYDLIRH or
QSYDMNVH.

In certain preferred embodiments, the antigen-binding domain includes a VL CDR1 sequence represented in the general formula

35 SGSnnNIgnNYVn

wherein each n independently represents any amino acid residue. For instance, the CDR1 sequence is SGSESNIGNNYVQ.

- In preferred embodiments, the mechanism of killing by multivalent forms of the subject compositions involves an innate pre-programmed process of said cell. For instance, the killing is non-apoptotic. Killing by the subject compositions can be dependent on the action of non-caspase proteases, and/or killing which cannot be inhibited by zVAD-fmk or zDEVD-fmk.
- 5
- 10 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide including at least a F(ab')₂ antibody fragment or a mini-antibody fragment.
- 15 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least two monovalent antibody fragments selected from Fv, scFv, dsFv and Fab fragments, and further comprises a cross-linking moiety or moieties.
- 20 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least one full antibody selected from the antibodies of classes IgG1, 2a, 2b, 3, 4, IgA, and IgM.
- 25 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide is formed prior to binding to said cell.
- 30 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide is formed after binding to said cell.
- 35 In certain preferred embodiments, the antigen binding sites are cross-linked to a polymer.
- Another aspect of the present invention provides a nucleic acid comprising a coding sequence for an antigen-binding domain, such as those antigen binding domains described above, or a multivalent polypeptide thereof. For example, in certain embodiments, the nucleic acid includes a coding sequence for a polypeptide comprising at least one antibody-based antigen-binding domain of human

composition with binding specificity for an antigen expressed on the surface of a human cell, wherein treating cells expressing the antigen with a multivalent form of the polypeptide causes or leads to killing of said cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain 5 embodiments, the nucleic acid includes a coding sequence for a polypeptide comprising at least one antibody-based antigen-binding domain which binds to at least one epitope of human HLA DR with a K_d of 1 μ M, 100nM, 10nM or even 1nM or less.

In certain embodiments, the nucleic acid includes a coding sequence for a polypeptide 10 comprising a plurality of antibody-based antigen-binding domains of human composition with binding specificity for human HLA DR, wherein treating a cell expressing HLA DR with the multivalent polypeptide causes or leads to killing of the cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In preferred embodiments, the antigen-binding domains individually bind to epitopes on the 15 human HLA DR with a K_d of 1 μ M, 100nM, 10nM or even 1nM or less.

In certain embodiments, the nucleic acid includes a coding sequence for a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition with binding specificity for human HLA DR, wherein treating a cell 20 expressing HLA DR with said multivalent polypeptide causes or leads to killing of said cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said cell killing. Preferably, the multivalent polypeptide has an EC₅₀ for killing killing activated lymphoid cells, transformed cells and/or lymphoid tumor cells of 100nM, 10nM or even 1nM or less.

25 Another aspect of the invention provides a vector comprising the coding sequence of any one of the subject nucleic acids, e.g., as described above, and a transcriptional regulatory sequence operably linked thereto.

30 Still another aspect of the present invention provides a host cell harboring at least one subject nucleic acids or the subject vector. Another aspect of the present invention provides a method for the production of a multivalent composition that causes or leads to killing of cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing comprising culturing the host 35 cells under conditions wherein the nucleic acid is expressed either as a polypeptide

comprising a plurality of antigen binding domains or as a polypeptide comprising at least one antigen binding domains which is subsequently treated to form a multivalent composition.

- 5 Another aspect of the present invention provides forms of the subject polypeptide or nucleic acid compositions, formulated in a pharmaceutically acceptable carrier and/or diluent. The present invention specifically contemplates the use of such compositions for preparing a pharmaceutical preparation for the treatment of animals, especially humans.
- 10 Such pharmaceutical compositions can be used for the treatment of conditions involving unwanted cell proliferation, particularly the treatment of a disorder involving transformed cells expressing MHC class II antigens. For instance, the formulations can be used for the treatment of a disorder selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy
15 cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, multiple myeloid leukemia and B cell precursor leukemia.

Such pharmaceutical preparations can be used for the treatment of diseases involving
20 unwanted activation of immune cells, such as in the treatment of a disorder selected from rheumatoid arthritis, juvenile arthritis, multiple sclerosis, Grave's disease, insulin-dependent diabetes, narcolepsy, psoriasis, systemic lupus erythematosus, ankylosing spondylitis, transplant rejection, graft vs. host disease, Hashimoto's disease, myasthenia gravis, pemphigus vulgaris, glomerulonephritis, thyroiditis, pancreatitis, insulitis, primary
25 biliary cirrhosis, irritable bowel disease and Sjogren syndrome.

Another aspect of the present invention provides a diagnostic composition including the polypeptide or nucleic acid compositions of the present invention. In certain embodiments, the diagnostic composition includes a polypeptide composition and a
30 cross-linking moiety or moieties.

Still another aspect of the present invention provides a method for killing a cell expressing an antigen on the surface of said cell comprising the step of contacting the cell with a multivalent polypeptide composition of the subject invention.

Another aspect of the invention provides a method to identify patients that can be treated with a multivalent polypeptide composition, formulated in a pharmaceutically acceptable carrier and/or diluent comprising the steps of

- a. Isolating cells from a patient;
- b. Contacting said cells with the composition; and
- c. Measuring the degree of killing or immunosuppression of said cells.

The present invention also provides a kit to identify patients that can be treated with a multivalent polypeptide composition of the present invention, formulated in a pharmaceutically acceptable carrier and/or diluent comprising

- a. a multivalent polypeptide composition; and
- b. Means to measure the degree of killing or immunosuppression of said cells.

In certain embodiments, the kit includes a multivalent polypeptide composition, and a cross-linking moiety. In other embodiments, the kit includes

- a. a multivalent polypeptide composition, and
- b. a detectable moiety or moieties, and
- c. reagents and/or solutions to effect and/or detect binding of (i) to an antigen.

Another aspect of the present invention provides a cytotoxic composition comprising a multivalent polypeptide composition operably linked to a cytotoxic agent.

Stil another aspect of the invention provides an immunogenic composition comprising a multivalent polypeptide composition operably linked to an immunogenic agent.

Another aspect of the present invention provides a method to kill a cell comprising contacting the cell with a multivalent polypeptide composition operably linked a cytotoxic or immunogenic agent.

Another aspect of the invention provides a method for treating a human to reduce the severity of disorder involving unwanted proliferation/activation of cells expressing the human β -chain of HLA DR, comprising administering to the patient a a multivalent polypeptide of the present invention. In certain embodiments, the disorder

involves unwanted proliferation/activation of lymphoid cells, e.g., selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid

5 leukemia, multiple myeloid leukemia and B cell precursor leukemia.

Another aspect of the invention provides a use of a multivalent polypeptide composition operably linked a cytotoxic or immunogenic agent for preparing a pharmaceutical preparation for the treatment of animals

10

According to a preferred embodiment, the polypeptide is directed to a lymphoid cell or a non-lymphoid cell that expresses MHC class II molecules. The latter type of cells occur for example at pathological sites of inflammation and/or autoimmune diseases, e.g. synovial cells, endothelial cells, thyroid stromal cells and glial cells, or it may also 15 comprise genetically altered cells capable of expressing MHC class II molecules.

Preferably, the polypeptide is directed to lymphoid tumor cells. More preferred are lymphoid tumor cells that represent a disease selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma,

20 Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia and B cell precursor leukemia. Most preferred are lymphoid tumor cells from a cell line taken from the list of GRANTA-519, PRIESS, KARPAS-422, DOHH-2, MHH-CALL-4, MN-60, BJAB, L-428, BONNA-12, EOL-1, MHH-PREB-1 and MHH-CALL-2 cell lines.

25 In certain embodiments, the polypeptide binds to at least one epitope in the alpha-chain of an HLA-DR molecule. In such embodiments, the polypeptide preferably binds to at least one epitope in the first domain of the alpha-chain of HLA-DR, the first domain being the N-terminal domain of the chain. For instance, the polypeptide can be selected to bind to at least one epitope within the alpha-helix ranging from Glu⁵⁵ to 30 Tyr⁷⁹ of the alpha-chain of HLA-DR.

In other embodiments, the polypeptide binds to at least one epitope in the beta-chain of an HLA-DR molecule. Preferably, the polypeptide binds to at least one epitope in the first domain of the beta-chain of HLA-DR, the first domain being the N-terminal

35 domain of the chain.

In certain embodiments, the mechanism of killing a target cell induced by the polypeptide involves an innate pre-programmed process of said cell. Preferably, the polypeptide induces a killing mechanism, which is not an apoptotic cell death process.

5

In a preferred embodiment the polypeptide induces a killing mechanism which is dependent on the action of proteases other than caspases, e.g., is a caspase-independent mechanism.

- 10 In a further embodiment the multivalent composition comprises at least one full antibody which is selected from classes IgG1, 2a, 2b, 3, 4, IgA, and IgM.

In a further embodiment the multivalent composition comprises at least one of a F(ab')₂ antibody fragment or mini-antibody fragment.

15

In a preferred embodiment the multivalent composition comprises at least two monovalent antibody fragments selected from Fv, scFv, dsFv and Fab fragments, and further comprises a cross-linking moiety or moieties.

- 20 The present invention also provides a composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for human HLA DR wherein binding of said polypeptide to said epitope causes or leads to suppression of the immune response and wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination
25 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

30

Another immunosuppressive composition of the present invention includes a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a K_d of 1μM, 100nM, 10nM or even 1nM or less, wherein treating cells expressing MHC class II antigen with the polypeptide causes or

leads to suppression of the immune response, e.g., preferably with an IC₅₀ of 1μM, 100nM, 10nM or even 1nM or less.

Another immunosuppressive composition of the present invention includes a polypeptide
5 comprising at least one antibody-based antigen-binding domain of human composition
with a binding specificity for a human MHC class II antigen with a K_d of 1μM , 100nM,
10nM or even 1nM or less, the antigen-binding domain being isolated by a method which
includes isolation of human VL and VH domains from a recombinant antibody display
library by ability to bind to human MHC class II antigen, wherein treating cells that
10 express MHC class II with said polypeptide causes or leads to suppression of the
immune response.

The subject immunosuppressive compositions can be generated using the antigen-
binding domain isolated by the further steps of:

- 15 a. generating a library of mutations at least one of the CDR1, CDR2 and
CDR3 domains of one or both of the VL and VH domains, and
b. isolation of VL and VH domains from the library of variants by ability to
bind to human MHC class II antigen with a K_d of 1μM or less.
- 20 In preferred embodiments, the antigen binding domains of the immunosuppressive
composition binds to HLA-DR, and preferably to the β-chain of HLA-DR, and even
more preferably to the first domain of the β-chain of HLA-DR.

In certain preferred embodiments, the immunosuppressive composition have an IC₅₀ for
25 suppressing the immune response of 1 μM, 100nM, 10nM or even 1nM or less.

In certain preferred embodiments, the immunosuppressive composition have an IC₅₀ for
inhibiting of IL-2 secretion of 1 μM, 100nM, 10nM or even 1nM or less.

30 In certain preferred embodiments, the immunosuppressive composition have an IC₅₀ for
inhibiting of T cell proliferation of 1 μM, 100nM, 10nM or even 1nM or less.

In certain preferred embodiments, the immunosuppressive composition have antigen-
binding domain that bind to an epitope of one or more HLA-DR types selected from the
35 group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402,

DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRw53-B4*0101 and DRw52-B3*0101, and in preferred embodiments, the antigen-binding domain binds to at least 5 different of said HLA-DR types (e.g., are pan-DR)

- 5 In certain embodiments, the immunosuppressive composition have antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-
10 GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

- In certain embodiments, the immunosuppressive composition have antigen-binding domain includes a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3
15 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and
wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

- 20 wherein each n independently represents any amino acid residue.

For instance, the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

- In certain embodiments, the immunosuppressive composition the antigen-binding
25 domain competes with antigen binding by an antibody having a VH CDR3 sequence represented by the general formula

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and
a VL CDR3 sequence represented by the general formula

QSYDnnnn

wherein each n independently represents any amino acid residue.

- In certain embodiments, the immunosuppressive composition the antigen-binding domain includes a VL CDR1 sequence represented in the general formula

35 SGSnnNIGnNYVn

wherein each n independently represents any amino acid residue. For example, the CDR1 sequence is SGSESNIGNNYVQ.

In certain embodiments, the subject immunosuppressive compositions suppress the
5 immune response by one or more of (a) down-regulation of expression of the antigen to
which the polypeptide binds; or (b) inhibiting of the interaction between said cell and other
cells, wherein said interaction would normally lead to an immune response.

Another aspect of the present invention provides nucleic acids which including a coding
10 sequence for an immunosuppressive polypeptide of the present invention. In certain
embodiments, the nucleic acid can be provided as part of a vector, e.g., including the
coding sequence and a transcriptional regulatory sequence operably linked thereto. The
nucleic acid and vectors of the present invention can be provided as part of a host cell,
e.g., which can be used to produce an immunosuppressive composition.

15 Another aspect of the present invention provides a method for suppressing activation
and/or proliferation of a lymphocyte, comprising contacting the cell with an
immunosuppressive polypeptide of the present invention.

20 The present invention also provides a pharmaceutical preparation comprising the a
polypeptide including an antibody-based antigen-binding domain with a binding specificity
for a human MHC class II antigen with a K_d of 1 μ M or less, e.g., in an amount sufficient
to suppress an immune response in an animal, inhibit IL-2 secretion in an animal, and/or
inhibit T cell proliferation in an animal.

25 Another aspect of the present invention relates to the use of a polypeptide including an
antibody-based antigen-binding domain with a binding specificity for a human MHC class
II antigen with a K_d of 1 μ M or less, for the preparation of a pharmaceutical composition
for the treatment of animals, such as where said animals are human.

30 The subject immunosuppressive pharmaceutical preparations can be used for
suppressing IL-2 secretion by a cell of the immune system. For example, these
preparations can be administered to the patient in an effective amount to reduce the level
of immunological responsiveness in the patient.

35

Still another aspect of the present invention provides a method for suppressing IL-2 secretion by a lymphocyte, comprising contacting the cell with an immunosuppressive polypeptide of the present invention.

5 The subject method can be used for immunosuppressing a human, e.g., by administering to the patient an effective amount of an immunosuppressive polypeptide of the present invention to reduce the level of immunological responsiveness.

10 The invention further relates to a diagnostic composition containing at least one polypeptide and/or nucleic acid according to the invention, optionally together with further reagents, such as buffers, for performing the diagnosis.

15 In a preferred embodiment the diagnostic composition contains the polypeptide according to the invention cross-linked by at least one moiety. Such moieties can be for example antibodies recognizing an epitope present on the polypeptide such as the FLAG peptide epitope (Hopp et al., 1988; Knappik and Plückthun, 1994) or bifunctional chemical compounds reacting with a nucleophilic amino acid side chain as present in cysteine or lysine (King et al., 1994). Methods for cross-linking polypeptides are well known to the practitioner of ordinary skill in the art.

20 A diagnostic composition containing at least one nucleic acid and/or variant thereof according to the invention is also contemplated.

25 Furthermore, the present invention relates to a kit comprising at least one polypeptide according to the present invention, and a cross-linking moiety.

30 Additionally, the present invention relates to a kit comprising (i) a polypeptide according to the present invention, (ii) a detectable moiety or moieties, and (iii) reagents and/or solutions to effect and/or detect binding of (i) to an antigen.

35 The present invention further relates to a multivalent composition comprising at least one polypeptide and comprising at least two antigen binding domains.

Still another aspect of the present invention provides a method for conducting a pharmaceutical business comprising:

- (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;
- (ii) generating a multivalent composition comprising a plurality of said antigen-binding domains, which multivalent composition kills with an EC₅₀ of 50nM or less transformed or activated cells where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing.;
- 5 (iii) conducting therapeutic profiling of the multivalent compositions for efficacy and toxicity in animals;
- (iv) preparing a package insert describing the multivalent composition for treatment of proliferative disorders; and
- 10 (v) marketing the multivalent composition for treatment of proliferative disorders.

The present invention also provides a method for conducting a life science business comprising:

- (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;
- (ii) generating a multivalent composition comprising a plurality of said antigen-binding domains, which multivalent composition kills with an EC₅₀ of 50nM or less transformed or activated cells where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing.;
- 20 (iii) licensing, jointly developing or selling, to a third party, the rights for selling the multivalent compositions.

25 In such embodiments, the the antigen-binding domain can be isolated by a method which includes

- a. isolation of VL and VH domains of human composition from a recombinant antibody display library by ability to bind to epitopes of HLA DR,
- b. generating a library of variants at least one of the CDR1, CDR2 and CDR3 domains of one or both of the VL and VH domains, and
- c. isolation of VL and VH domains from the library of variants by ability to epitopes of HLA DR with a K_d of 1μM or less.

30 Another business method contemplated by the present invention includes:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a K_d of 1μM or less;

- (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an IC₅₀ of 100nM or less;
- (iii) conducting therapeutic profiling of the multivalent compositions for efficacy and toxicity in animals;
- 5 (iv) preparing a package insert describing the use of the composition for immunosuppression therapy; and
- (v) marketing the multivalent composition for use as an immunosuppressant.

The present invention also provides a method for conducting a life science business comprising:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a K_d of 1μM or less;
- (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an IC₅₀ of 100nM or less;
- 15 (iii) licensing, jointly developing or selling, to a third party, the rights for selling the compositions.

As used herein, the term "peptide" relates to molecules consisting of one or more chains of multiple, i. e. two or more, amino acids linked via peptide bonds.

20 The term "protein" refers to peptides where at least part of the peptide has or is able to acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its peptide chain(s). This definition comprises proteins such as naturally occurring or at least partially artificial proteins, as well as fragments or domains of whole proteins, as long as these fragments or domains are able to acquire a defined three-dimensional arrangement as described above.

25 The term "polypeptide" is used interchangeably to refer to peptides and/or proteins. Moreover, the terms "polypeptide" and "protein", as the context will admit, include multi-chain protein complexes, such as immunoglobulin polypeptides having separate heavy and light chains.

30 In this context, "polypeptide comprising at least one antibody-based antigen-binding domain" refers to an immunoglobulin (or antibody) or to a fragment thereof. The term

"fragment", with respect to antibody domains and the like, refers to a fragment of an immunoglobulin which retains the antigen-binding moiety of an immunoglobulin. Functional immunoglobulin fragments according to the present invention may be Fv (Skerra and Plückthun, 1988), scFv (Bird et al., 1988; Huston et al., 1988), disulfide-linked Fv (Glockshuber et al., 1992; Brinkmann et al., 1993), Fab, F(ab')₂ fragments or other fragments well-known to the practitioner skilled in the art, which comprise the variable domains of an immunoglobulin or functional immunoglobulin fragment.

Examples of polypeptides consisting of one chain are single-chain Fv antibody fragments, and examples for polypeptides consisting of multiple chains are Fab antibody fragments.

The term "antibody" as used herein, unless indicated otherwise, is used broadly to refer to both antibody molecules and a variety of antibody derived molecules. Such antibody derived molecules comprise at least one variable region (either a heavy chain or light chain variable region) and include such fragments as described above, as well as individual antibody light chains, individual antibody heavy chains, chimeric fusions between antibody chains and other molecules, and the like.

The "antigen-binding site" of an immunoglobulin molecule refers to that portion of the molecule that is necessary for binding specifically to an antigen. An antigen binding site preferably binds to an antigen with a Kd of 1μM or less, and more preferably less than 100nM, 10nM or even 1nM in certain instances. Binding specifically to an antigen is intended to include binding to the antigen which significantly higher affinity than binding to any other antigen.

The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to

each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

5

For the purposes of this application, "valent" refers to the number of antigen binding sites the subject polypeptide possess. Thus, a bivalent polypeptide refers to a polypeptide with two binding sites. The term "multivalent polypeptide" encompasses bivalent, trivalent, tetravalent, etc. forms of the polypeptide.

10

As used herein, a "multivalent composition" means a composition comprising a polypeptide having at least two of said antigen-binding domains, e.g., a multivalent polypeptide. Preferably, said at least two antigen-binding domains are in close proximity so as to mimic the structural arrangement relative to each other of binding sites comprised in a full immunoglobulin molecule. Examples for multivalent compositions are full immunoglobulin molecules (e.g. IgG, IgA or IgM molecules) or multivalent fragments thereof (e.g. F(ab')₂). Additionally, multivalent compositions of higher valencies may be formed from two or more multivalent compositions (e.g. two or more full immunoglobulin molecules), e.g. by cross-linking. Multivalent compositions, however, may be formed as well from two or more monovalent immunoglobulin fragments, e.g. by self-association as in mini-antibodies, or by cross-linking.

25

Accordingly, an "antibody-based antigen-binding domain" refers to polypeptide or polypeptides which form an antigen-binding site retaining at least some of the structural features of an antibody, such as at least one CDR sequence. In certain preferred embodiments, antibody-based antigen-binding domain includes sufficient structure to be considered a variable domain, such as three CDR regions and interspersed framework regions. Antibody-based antigen-binding domain can be formed single polypeptide chains corresponding to VH or VL sequences, or by intermolecular or intramolecular association of VH and VL sequences.

30

The term "recombinant antibody library" describes a variegated library of antigen binding domains. For instance, the term includes a collection of display packages,

e.g., biological particles, which each have (a) genetic information for expressing at least one antigen binding domain on the surface of the particle, and (b) genetic information for providing the particle with the ability to replicate. For instance, the package can display a fusion protein including an antigen binding domain. The
5 antigen binding domain portion of the fusion protein is presented by the display package in a context which permits the antigen binding domain to bind to a target epitope that is contacted with the display package. The display package will generally be derived from a system that allows the sampling of very large variegated antibody libraries. The display package can be, for example, derived from vegetative bacterial
10 cells, bacterial spores, and bacterial viruses.

In an exemplary embodiment of the present invention, the display package is a phage particle which comprises a peptide fusion coat protein that includes the amino acid sequence of a test antigen binding domains. Thus, a library of replicable phage
15 vectors, especially phagemids (as defined herein), encoding a library of peptide fusion coat proteins is generated and used to transform suitable host cells. Phage particles formed from the chimeric protein can be separated by affinity selection based on the ability of the antigen binding site associated with a particular phage particle to specifically bind a target epitope. In a preferred embodiment, each individual phage
20 particle of the library includes a copy of the corresponding phagemid encoding the peptide fusion coat protein displayed on the surface of that package. Exemplary phage for generating the present variegated peptide libraries include M13, f1, fd, If1, IKe, Xf, Pf1, Pf3, λ, T4, T7, P2, P4, φX-174, MS2 and f2.

25 The term "generating a library of variants of at least one of the CDR1, CDR2 and CDR3" refers to a process of generating a library of variant antigen binding sites in which the members of the library differ by one or more changes in CDR sequences, e.g., not FR sequences. Such libraries can be generated by random or semi-random mutagenesis of one or more CDR sequences from a selected antigen binding site.
30

As used herein, an "antibody-based antigen-binding domain of human composition" preferably means a polypeptide comprising at least an antibody VH domain and an antibody VL domain, wherein a homology search in a database of protein sequences comprising immunoglobulin sequences results for both the VH and the VL domain in

an immunoglobulin domain of human origin as hit with the highest degree of sequence identity. Such a homology search may be a BLAST search, e.g. by accessing sequence databases available through the National Center for Biological Information and performing a "BasicBLAST" search using the "blastp" routine. See
5 also Altschul et al. (1990) J Mol Biol 215:403-410. Preferably, such a composition does not result in an adverse immune response thereto when administered to a human recipient. In certain preferred embodiments, the subject antigen-binding domains of human composition include the framework regions of native human immunoglobulins, as may be cloned from activated human B cells, though not
10 necessarily all of the CDRs of a native human antibody.

As used herein, the term "mini-antibody fragment" means a multivalent antibody fragment comprising at least two antigen-binding domains multimerized by self-associating domains fused to each of said domains (Pack, 1994), e.g. dimers
15 comprising two scFv fragments, each fused to a self-associating dimerization domain. Dimerization domains, which are particularly preferred, include those derived from a leucine zipper (Pack and Plückthun, 1992) or helix-turn-helix motif (Pack et al., 1993).

As used herein, "activated cells" means cells of a certain population of interest, which
20 are not resting. Activation might be caused by mitogens (e.g., lipoproteinsaccharide, phytohemagglutinine) or cytokines (e.g., interferon gamma). Preferably, said activation occurs during tumor transformation (e.g., by Epstein-Barr virus, or "spontaneously"). Preferably, activated cells are characterized by the features of MHC class II molecules expressed on the cell surface and one or more additional
25 features including increased cell size, cell division, DNA replication, expression of CD45 or CD11 and production/secretion of immunoglobulin.

As used herein, "non-activated cells" means cells of a population of interest, which
30 are resting and non-dividing. Said non-activated cells may include resting B cells as purified from healthy human blood. Such cells can, preferably, be characterized by lack or reduced level of MHC class II molecules expressed on the cell surface and lack or reduced level of one or more additional features including increased cell size, cell division, DNA replication, expression of CD45 or CD11 and production/secretion of immunoglobulin.

As used herein, the term "EC50" means the concentration of multivalent forms of the subject compositions which produces 50% of its maximum response or effect, such as cell killing.

5

"At least 5-fold lower EC50" means that the concentration of a multivalent composition comprising at least one polypeptide of the present invention that is required to kill 50% of activated cells is at least five times less than the concentration of the multivalent composition required to kill non-activated cells. Preferably,

10 the concentration required to kill 50% of non-activated cells cannot be achieved with therapeutically appropriate concentrations of the multivalent composition. Most preferably, the EC50 value is determined in the test described below in the appended examples.

15 The term "immunosuppress" refers to the prevention or diminution of the immune response, as by irradiation or by administration of antimetabolites, antilymphocyte serum, or specific antibody.

20 The term "immune response" refers to any response of the immune system, or a cell forming part of the immune system (lymphocytes, granulocytes, macrophages, etc), to an antigenic stimulus, including, without limitation, antibody production, cell-mediated immunity, and immunological tolerance.

25 As used herein, the term "IC50" with respect immuno suppression, refers to the concentration of the subject compositions which produces 50% of its maximum response or effect, such as inhibition of an immune response, such as may be manifest by inhibition of IL2 secretion, down-regulation of IL2 expression, or reduced rate of cell proliferation.

30 The phrase "cytotoxic entities", with reference to a manner of cell killing, refers to mechanisms which are complement-dependent. Likewise, the phrase "immunological mechanism", with reference to a manner of cell killing, refers to macrophage-dependent and/or neutrophil-dependent killing of cells.

"Lymphoid cells" when used in reference to a cell line or a cell, means that the cell line or cell is derived from the lymphoid lineage. "Lymphoid cells" include cells of the B and the T lymphocyte lineages, and of the macrophage lineage.

- 5 Cells, which are "non lymphoid cells and express MHC class II", are cells other than lymphoid cells that express MHC class II molecules, e.g. during a pathological inflammatory response. For example, said cells may include synovial cells, endothelial cells, thyroid stromal cells and glial cells, and it may also comprise genetically altered cells capable of expressing MHC class II molecules.

10

The terms "apoptosis" and "apoptotic activity" refer to the form of cell death in mammals that is accompanied by one or more characteristic morphological and biochemical features, including nuclear and condensation of cytoplasm, chromatin aggregation, loss of plasma membrane microvilli, partition of cytoplasm and nucleus

- 15 into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material, degradation of chromosomal DNA or loss of mitochondrial function. Apoptosis follows a very stringent time course and is executed by caspases, a specific group of proteases. Apoptotic activity can be determined and measured, for instance, by cell viability assays, Annexin V staining or caspase inhibition assays. Apoptosis can be induced 20 using a cross-linking antibody such as anti-CD95 as described in Example H.

As used herein, the term "first domain of the α -chain of HLA-DR" means the N-terminal domain of the alpha-chain of the MHC class II DR molecule.

25

As used herein, the term "first domain of the β -chain of HLA-DR" means the N-terminal domain of the beta-chain of the MHC class II DR molecule.

- 30 The term "innate pre-programmed process" refers to a process that, once it is started, follows an autonomous cascade of mechanisms within a cell, which does not require any further auxillary support from the environment of said cell in order to complete the process.

As used herein, the term "HuCAL" refers to a fully synthetic human combinatorial antibody library as described in Knappik et al. (2000).

The term "variable region" as used herein in reference to immunoglobulin molecules
5 has the ordinary meaning given to the term by the person of ordinary skill in the act of immunology. Both antibody heavy chains and antibody light chains may be divided into a "variable region" and a "constant region". The point of division between a variable region and a heavy region may readily be determined by the person of ordinary skill in the art by reference to standard texts describing antibody structure,
10 e.g., Kabat et al "Sequences of Proteins of Immunological Interest: 5th Edition" U.S. Department of Health and Human Services, U.S. Government Printing Office (1991).

As used herein, the term "CDR3" refers to the third complementarity-determining region of the VH and VL domains of antibodies or fragments thereof, wherein the VH
15 CDR3 covers positions 95 to 102 (possible insertions after positions 100 listed as 100a to 100z), and VL CDR3 positions 89 to 96 (possible insertions in V λ after position 95 listed as 95a to 95c) (see Knappik et al., 2000).

As used herein, the term "hybridizes under stringent conditions" is intended to
20 describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least 65%, more preferably at least 70%, and even more preferably at least 75% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled
25 in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, New York. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions is hybridization in 6 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 x SSC, 0.1% SDS at 50°-65°C.

30 A "protein coding sequence" or a sequence which "encodes" a particular polypeptide or peptide, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a

translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding
5 sequence.

Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences which encode a polypeptide, as the term is typically used, as well as DNA sequences which are transcribed into inhibitory antisense molecules.

10

As used herein, the term "transfection" means the introduction of a heterologous nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is
15 transcribed into mRNA and translated into protein. A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct is capable of being inherited by daughter cells.

20

"Expression vector" refers to a replicable DNA construct used to express DNA which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (1) agent(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a DNA sequence encoding a desired protein (such as a polypeptide of the present invention) which is transcribed into mRNA and translated into protein, and (3) appropriate transcription
25 and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used
30 form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

In the expression vectors, regulatory elements controlling transcription or translation can be generally derived from mammalian, microbial, viral or insect genes. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated.

5 Vectors derived from viruses, such as retroviruses, adenoviruses, and the like, may be employed.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and
10 promoters and the like which induce or control transcription of protein coding sequences with which they are operably linked. It will be understood that a recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of the gene, if any.

15 "Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a promoter or other transcriptional regulatory sequence is operably linked to a coding sequence if it controls the transcription of the coding sequence.

20 As used herein, the term "fusion protein" is art recognized and refer to a chimeric protein which is at least initially expressed as single chain protein comprised of amino acid sequences derived from two or more different proteins, e.g., the fusion protein is a gene product of a fusion gene.

25 As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

The "growth rate" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

30 The term "cell-proliferative disorder" denotes malignant as well as nonmalignant populations of transformed cells which morphologically often appear to differ from the surrounding tissue.

As used herein, "transformed cells" refers to cells which have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with
5 respect to their loss of growth control.

As used herein, "immortalized cells" refers to cells which have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

10

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

15 According to the methods of the invention, the peptide may be administered in a pharmaceutically acceptable composition. In general, pharmaceutically-acceptable carriers for monoclonal antibodies, antibody fragments, and peptides are well-known to those of ordinary skill in the art. As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings,
20 antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. In preferred embodiments, the subject carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not excessively toxic to the hosts of the concentrations of which it is administered.
25 The administration(s) may take place by any suitable technique, including subcutaneous and parenteral administration, preferably parenteral. Examples of parenteral administration include intravenous, intraarterial, intramuscular, and intraperitoneal, with intravenous being preferred.

30 The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or

dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required 5 particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable 10 compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds, e.g., the subject polypeptides, in the required amount in the appropriate solvent with 15 various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred 20 methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For oral administration the polypeptides of the present invention may be incorporated 25 with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically 30 effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free

amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, 5 calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with 10 sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of 15 hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human 20 administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Upon formulation, solutions can be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The 25 formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, the term "prophylactic or therapeutic" treatment refers to administration to the host of the medical condition. If it is administered prior to 30 exposure to the condition, the treatment is prophylactic (i.e., it protects the host against tumor formation), whereas if administered after initiation of the disease, the treatment is therapeutic (i.e., it combats the existing tumor).

A multivalent composition of at least one polypeptide according to the invention is capable of causing cell death of activated cells, preferably lymphoid tumor cells without requiring any further additional measures such as chemotherapy and with limited immunogenic side effects on the treated patient. Further, the multivalent
5 composition comprising a polypeptide according to the invention has the capability of binding to at least one epitope on the target antigen, however, several epitope binding sites might be combined in one molecule. Preferably, the multivalent composition comprising a polypeptide according to the invention shows at least 5-fold, or more preferably 10-fold higher killing activity against activated cells compared to non-
10 activated cells. This higher activity on activated cells can be expressed as the at least 5-fold lower EC50 value on activated versus non-activated cells or as the higher percentage of killing of activated cells versus non-activated cells when using the same concentration of protein. Under the latter alternative, the multivalent composition comprising a polypeptide according to the invention at a given
15 polypeptide concentration kills at least 50%, preferably at least 80%, of activated cells, whereas the same concentration of a multivalent composition comprising a polypeptide according to the invention under the same incubation conditions kills less than 15%, preferably less than 10% of the non-activated cells. The assay conditions for determining the EC50 value and the percentage killing activity are described
20 below.

Brief Description of the Drawings

Figure 1

- 25 a. Specificity of the anti-HLA-DR antibody fragments: Binding of MS-GPC-8-27-7, MS-GPC-8-27-10, MS-GPC-8-6-13, MS-GPC-8-27-41, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-6-27, MS-GPC-8 and MS-GPC-8-6 to HLA-DR protein, negative control proteins (BSA, testosterone-BSA, lysozyme and human apotransferrin), and an empty microtiter plate well (plastic). Specificity was assessed using standard
30 ELISA procedures.
- b. Specificity of the anti-HLA-DR antibody fragments MS-GPC-1, 6, 8 & 10 isolated from the HuCAL library to HLA-DR protein, a mouse-human chimeric HLA protein and negative control proteins (lysozyme, transferrin, BSA and human β -globulin).

Specificity was assessed using standard ELISA procedures. A non-related antibody fragment (irr. scFv) was used as control.

Figure 2

5 Reactivity of the anti-HLA-DR antibody fragments (MS-GPC-1, 6, 8 and 10) and IgG forms of MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-41 & MS-GPC-8-6-17 to various cell lines expressing MHC class II molecules. "+" represents strong reactivity as detected using standard immunofluorescence procedure. "+/—" represents weak reactivity and "-" represents no detected reactivity between an anti-HLA-DR antibody
10 fragment or IgG and a particular cell line.

Figure 3

Viability of tumor cells in the presence of monovalent and cross-linked anti-HLA-DR antibody fragments as assessed by trypan blue staining. Viability of GRANTA-519
15 cells was assessed after 4 h incubation with anti-HLA-DR antibody fragments (MS-GPC-1, 6, 8 and 10) with and without anti-FLAG M2 mAb as cross-linking agent.

Figure 4

Scatter plots and fitted logistic curves of data from Table 5 showing improved killing
20 efficiency of 50 nM solutions of the IgG form of the human antibody fragments of the invention treated compared to treatment with 200 nM solutions of murine antibodies. Open circles represent data for cell lines treated with the murine antibodies L243 and 8D1 and closed circles for human antibodies MS-GPC-8, MS-GPC-8-27-41, MS-GPC-8-10-57 and MS-GPC-8-6-13. Fitted logistic curves for human (solid) and
25 mouse (dashed) mAb cell killing data show the overall superiority of the treatment with human mAbs at 50 nM compared to the mouse mAbs despite treatment at a final concentration of 200 nM.

30 **Figure 5**

Killing of activated versus non-activated cells. MHH-PREB-1 cells are activated with Lipopolysaccharide, Interferon-gamma and phyto-hemagglutin, and subsequently incubated for 4 h with 0.07 to 3300 nM of the IgG forms of the anti-HLA-DR antibody

fragments MS-GPC-8-10-57 and MS-GPC-8-27-41. No loss of viability in the control non-activated MHH-PREB-1 cells is seen.

Figure 6

5 Killing efficiency of control (no antibody, unreactive murine IgG; light grey), and
human (MS-GPC-8, MS-GPC-8-10-57 & MS-GPC-8-27-41; dark grey) IgG forms of
anti-HLA-DR antibody fragments against CLL cells isolated from patients. Left panel,
box-plot display of viability data from 10 patient resting cell cultures against antibodies
10 after incubation for four (h4) and twenty four hours (h24). Right panel box-plot display
of viability data from 6 patient activated cell cultures against antibodies after
incubation for four (h4) and twenty four hours (h24).

Figure 7

15 Concentration dependent cell viability for certain anti-HLA-DR antibody fragments of
the invention. Vertical lines indicate the EC50 value estimated by logistic non-linear
regression on replica data obtained for each of the antibody fragments. a) Killing
curves of cross-linked bivalent anti-HLA-DR antibody F(ab) fragment dimers MS-
GPC-10 (circles and solid line), MS-GPC-8 (triangles and dashed line) and MS-GPC-
20 1 (crosses and dotted line). b) Killing curves of cross-linked bivalent anti-HLA-DR
antibody (Fab) fragment dimers MS-GPC-8-17 (circles and solid line), and murine
IgGs 8D1 (triangles and dashed line) and L243 (crosses and dotted line). c) Killing
curves of cross-linked bivalent anti-HLA-DR antibody (Fab) fragment dimers GPC-8-
25 6-2 (crostriangles and dashed line), and murine IgGs 8D1 (circles and solid line) and
L243 (crosses and dotted line). d) Killing curves of IgG forms of human anti-HLA-DR
antibody fragments MS-GPC-8-10-57 (crosses and dotted line), MS-GPC-8-27-41
(exes and dash-dot line), and murine IgGs 8D1 (circles and solid line) and L243
(triangles and dashed line). All concentrations are given in nM of the bivalent agent
(IgG or cross-linked (Fab) dimer).

30

Figure 8

a. Incubation of Priess cells with the anti-HLA-DR antibody fragment MS-GPC-8,
cross-linked using the anti-FLAG M2 mAb, shows more rapid killing than a culture of

Priess cells induced into apoptosis using anti-CD95 mAb. An Annexin V/PI staining technique identifies necrotic cells by Annexin V positive and PI positive staining.

b. Incubation of Priess cells with the anti-HLA-DR antibody fragment MS-GPC-8, 5 cross-linked using the anti-FLAG M2 mAb, shows little evidence of an apoptotic mechanism compared to an apoptotic culture of Priess cells induced using anti-CD95 mAb. An Annexin V/PI staining technique identifies apoptotic cells by Annexin V positive and PI negative staining.

10 **Figure 9**

a. Immunosuppressive properties of the IgG forms of the anti-HLA-DR antibody fragments MS-GPC-8-10-57, MS-GPC-8-27-41 & MS-GPC-8-6-13 using an assay to determine inhibition of IL-2 secretion from T-hybridoma cells.
b. Immunosuppressive properties of the monovalent Fab forms of the anti-HLA-DR 15 antibody fragments MS-GPC-8-27-41 & MS-GPC-8-6-19 using an assay to determine inhibition of IL-2 secretion from T-hybridoma cells

Figure 10

Immunosuppressive properties of the IgG forms of the anti-HLA-DR antibody 20 fragments MS-GPC-8-10-57 and MS-GPC-8-27-41 in an assay to determine inhibition of T cell proliferation.

Figure 11

Vector map and sequence of scFv phage display vector pMORPH13_scFv.
25 The vector pMORPH13_scFv is a phagemid vector comprising a gene encoding a fusion between the C-terminal domain of the gene III protein of filamentous phage and a HuCAL scFv. In Figure 11, a vector comprising a model scFv gene (combination of VH1A and Vλ3 (Knappik et al., 2000) is shown.
The original HuCAL master genes (Knappik et al. (2000): see Fig. 3 therein) have 30 been constructed with their authentic N-termini: VH1A, VH1B, VH2, VH4 and VH6 with Q (=CAG) as the first amino acid. VH3 and VH5 with E (=GAA) as the first amino acid. Vector pMORPH13_scFv comprises the short FLAG peptide sequence (DYKD) fused to the VH chain, and thus all HuCAL VH chains in, and directly derived from,

this vector have E (=GAA) at the first position (e.g. in pMx7_FS vector, see Figure 12).

Figure 12

5 Vector map and sequence of scFv expression vector pMx7_FS_5D2.

The expression vector pMx7_FS_5D2 leads to the expression of HuCAL scFv fragments (in Figure 12, the vector comprises a gene encoding a "dummy" antibody fragment called "5D2") when VH-CH1 is fused to a combination of a FLAG tag (Hopp et al., 1988; Knappik and Plückthun, 1994) and a STREP tag II (WSHPQFEK) (IBA 10 GmbH, Göttingen, Germany; see: Schmidt and Skerra, 1993; Schmidt and Skerra, 1994; Schmidt et al., 1996; Voss and Skerra, 1997).

Figure 13

Vector map and sequence of Fab expression vector pMx9_Fab_GPC8.

15 The expression vector pMx9_Fab_GPC8 leads to the expression of HuCAL Fab fragments (in Figure 13, the vector comprises the Fab fragment MS-GPC8) when VH-CH1 is fused to a combination of a FLAG tag (Hopp et al., 1988; Knappik and Plückthun, 1994) and a STREP tag II (WSHPQFEK) (IBA GmbH, Göttingen, Germany; see: Schmidt and Skerra, 1993; Schmidt and Skerra, 1994; Schmidt et al., 20 1996; Voss and Skerra, 1997).

In pMx9_Fab vectors, the HuCAL Fab fragments cloned from the scFv fragments (see figure caption of Figure 11) do not have the short FLAG peptide sequence (DYKD) fused to the VH chain, and all HuCAL VH chains in, and directly derived from, that vector have Q (=CAG) at the first position

25

Figure 14

Vector map and sequence of Fab phage display vector pMORPH18_Fab_GPC8.

30 The derivatives of vector pMORPH18 are phagemid vectors comprising a gene encoding a fusion between the C-terminal domain of the gene III protein of filamentous phage and the VH-CH1 chain of a HuCAL antibody. Additionally, the vector comprises the separately encoded VL-CL chain. In Figure 14, a vector comprising the Fab fragment MS-GPC-8 is shown.

In pMORPH18_Fab vectors, the HuCAL Fab fragments cloned from the scFv fragments (see figure caption of Figure 11) do not have the short FLAG peptide

sequence (DYKD) fused to the VH chain, and all HuCAL VH chains in, and directly derived from, that vector have Q (=CAG) at the first position.

Figure 15

5 Amino acid sequences of VH and VL domains of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-6, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-27, MS-GPC-8-6-13, MS-GPC-8-10-57, and MS-GPC-8-27-41.

The sequences in Figure 15 show amino acid 1 of VH as constructed in the original HuCAL master genes (Knappik et al. (2000): see Fig. 3 therein). In scFv constructs, 10 as described in this application, amino acid 1 of VH is always E (see figure caption of Figure 11), in Fab constructs as described in this application, amino acid 1 of VH is always Q (see figure caption of Figure 13)

Detailed Description of the Invention

15 The following examples illustrate the invention.

Examples

All buffers, solutions or procedures without explicit reference can be found in standard textbooks, for example Current Protocols of Immunology (1997 and 1999) or 20 Sambrook et al., 1989. Where not given otherwise, all materials were purchased from Sigma, Deisenhofen, DE, or Merck, Darmstadt, DE, or sources are given in the literature cited. Hybridoma cell lines LB3.1 and L243 were obtained from LGC Reference Materials, Middlesex, UK; data on antibody 8D1 were generously supplied by Dr. Matyas Sandor, University of Michigan, Madison, WI, USA.

25

1. Preparation of a human antigen

To demonstrate that we could identify cytotoxic antigen-binding domains of human composition, we first prepared a purified form of a human antigen, the human MHC class II DR protein (DRA*0101/DRB1*0401) from PRIESS cells (Gorga et al., 1984; 30 Gorga et al., 1986; Gorga et al., 1987; Stern et al., 1992) as follows.

First, PRIESS cells (ECACC, Salisbury UK) were cultured in RPMI and 10% fetal calf serum (FCS) using standard conditions, and 10^{10} cells were lysed in 200 ml phosphate buffered saline (PBS) (pH 7.5) containing 1% NP-40 (BDH, Poole, UK), 25

mM iodoacetamide, 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mg/l each of the protease inhibitors chymostatin, antipain, pepstatin A, soybean trypsin inhibitor and leupeptin. The lysate was centrifuged at 10.000 g (30 minutes, 4°C) and the resulting supernatant was supplemented with 40 ml of an aqueous solution
5 containing 5% sodium deoxycholate, 5 mM iodoacetamide and 10 mg/l each of the above protease inhibitors and centrifuged at 100.000 g for two hours (4°C). To remove material that bound non-specifically and endogenous antibodies, the resulting supernatant was made 0.2 mM with PMSF and passed overnight (4°C) through a rabbit serum affigel-10 column (5 ml; for preparation, rabbit serum (Charles River,
10 Wilmington, MA, USA) was incubated with Affigel 10 (BioRad, Munich, DE) at a volume ratio of 3:1 and washed following manufacturer's directions) followed by a Protein G Sepharose Fast Flow column (2 ml; Pharmacia) using a flow rate of 0.2 ml/min.

15 Second, the pre-treated lysate was batch incubated with 5 ml Protein G Sepharose Fast Flow beads coupled to the murine anti-HLA-DR antibody LB3.1 (obtained by Protein G-Sepharose FF (Pharmacia) affinity chromatography of a supernatant of hybridoma cell line LB3.1) (Stern et al., 1993) overnight at 4°C using gentle mixing, and then transferred into a small column which was then washed extensively with
20 three solutions: (1) 100 ml of a solution consisting of 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 10% glycerol and 0.03% sodium azide at a flow rate of 0.6 ml/min. (2) 25 ml of a solution consisting of 50 mM Tris/HCl (pH 9.0), 0.5 M NaCl, 0.5 % NP-40, 0.5% sodium deoxycholate, 10% glycerol and 0.03% sodium azide at a flow rate of 0.9 ml/min; (3) 25 ml of a solution
25 consisting of 2 mM Tris/HCl (pH 8.0), 1% octyl- β -D-glucopyranoside, 10% glycerol and 0.03% sodium azide at a flow rate of 0.9 ml/min.

Third, MHC class II DR protein (DRA*0101/DRB1*0401) was eluted using 15 ml of a solution consisting of 50 mM diethylamine/HCl (pH 11.5), 150 mM NaCl, 1 mM EDTA,
30 1 mM EGTA, 1% octyl- β -D-glucopyranoside (Alexis Corp., Lausen, CH), 10% glycerol, 10 mM iodoacetamide and 0.03% sodium azide at a flow rate of 0.4 ml/min. 800 μ l fractions were immediately neutralised with 100 μ l 1M Tris/HCl (pH 6.8), 150 mM NaCl and 1% octyl- β -D-glucopyranoside. The incubation of the lysate with LB3.1-Protein G Sepharose Fast Flow beads was repeated until the lysate was exhausted of

MHC protein. Pure eluted fractions of the MHC class II DR protein (as analyzed by SDS-PAGE) were pooled and concentrated to 1.0-1.3 g/l using Vivaspin concentrators (Greiner, Solingen, DE) with a 30 kDa molecular weight cut-off. Approximately 1 mg of the MHC class II DR preparation was re-buffered with PBS
5 containing 1% octyl- β -D-glucopyranoside using the same Vivaspin concentrator to enable direct coupling of the protein to BIACore CM5 chips.

2. Screening of HuCAL

2.1. Introduction

10 We identified certain antigen binding antibody fragments of human composition (MS-GPC-1, MS-GP-6, MS-GPC-8 and MS-GPC-10) against the human antigen (DRA*0101/DRB1*0401) from a human antibody library based on a novel concept that has been recently developed (Knappik et al., 2000). A consensus framework resulting in a total of 49 different frameworks here represents each of the VH- and
15 VL-subfamilies frequently used in human immune responses. These master genes were designed to take into account and eliminate unfavorable residues promoting protein aggregation as well as to create unique restriction sites leading to modular composition of the genes. In HuCAL-scFv, both the VH- and VL-CDR3 encoding regions of the 49 master genes were randomized.
20

2.2. Phagemid rescue, phage amplification and purification

The HuCAL-scFv (Knappik et al., 2000) library, cloned into a phagemid-based phage display vector pMORPH13_scFv (see Figure 11), in *E.coli* TG-1 was amplified in 2 x TY medium containing 34 μ g/ml chloramphenicol and 1% glucose (2 x TY-CG). After
25 helper phage infection (VCSM13) at 37°C at an OD₆₀₀ of about 0.5, centrifugation and resuspension in 2 x TY / 34 μ g/ml chloramphenicol / 50 μ g/ml kanamycin / 0.1 mM IPTG, cells were grown overnight at 30°C. Phage were PEG-precipitated from the supernatant (Ausubel et al., 1998), resuspended in PBS/20% glycerol and stored at –80°C. Phage amplification between two panning rounds was conducted as follows:
30 mid-log phase TG1-cells were infected with eluted phage and plated onto LB-agar supplemented with 1% of glucose and 34 μ g/ml of chloramphenicol. After overnight incubation at 30°C colonies were scraped off, adjusted to an OD₆₀₀ of 0.5 and helper phage added as described above.

2.3. Manual solid phase panning

Wells of MaxiSorp™ microtiterplates (Nunc, Roskilde, DK) were coated with MHC-class II DRA*0101/DRB1*0401 (prepared as above) dissolved in PBS (2 µg/well).

After blocking with 5% non-fat dried milk in PBS, 1–5 × 10¹² HuCAL-scFv phage
5 purified as above were added for 1h at 20°C. After several washing steps, bound phages were eluted by pH-elution with 100 mM triethylamine and subsequent neutralization with 1M TRIS-Cl pH 7.0. Three rounds of panning were performed with phage amplification conducted between each round as described above.

10 2.4. Mixed solid phase/whole cell panning

Three rounds of panning and phage amplification were performed as described in 2.3. and 2.2. with the exception that in the second round between 1 × 10⁷ and 5 × 10⁷ PRIESS cells in 1 ml PBS/10% FCS were used in 10 ml Falcon tubes for whole cell panning. After incubation for 1h at 20°C with the phage preparation, the cell
15 suspension was centrifuged (2000 rpm for 3 min) to remove non-binding phage, the cells were washed three times with 10 ml PBS, each time followed by centrifugation as described. Phage that specifically bound to the cells were eluted off by pH-elution using 100 mM HCl. Alternatively, binding phage could be amplified by directly adding E.coli to the suspension after triethylamine treatment (100 mM) and subsequent
20 neutralization.

2.5 Identification of HLA-DR binding scFv fragments

Clones obtained after three rounds of solid phase panning (2.3) or mixed solid phase/whole cell panning (2.4) were screened by FACS analysis on PRIESS cells for
25 binding to HLA-DR on the cell surface. For expression, the scFv fragments were cloned via XbaI/EcoRI into pMx7_FS as expression vector (see Figure 12). Expression conditions are shown below in example 3.2

Aliquots of 10⁶ Priess cells were transferred at 4°C into wells of a 96-well
30 microtiterplate. ScFv in blocking buffer (PBS/5% FCS) were added for 60 min and detected using an anti-FLAG M2 antibody (Kodak) (1:5000 dilution) followed by a polyclonal goat anti-mouse IgG antibody-R-Phycoerythrin-conjugate (Jackson ImmunoResearch, West Grove, PA, USA, Cat. No. 115-116-146, F(ab')₂ fragment) (1:200 dilution). Cells were fixed in 4% paraformaldehyde for storage at 4°C. 10⁴

events were collected for each assay on the FACS-Calibur (BD Immunocytometry Systems, San Jose, CA, USA).

Only fifteen out of over 500 putative binders were identified which specifically bound to Priess cells. These clones were further analyzed for their killing activity as described below. Table 1 contains the sequence characteristics of clones MS-GPC-1, 5 MS-GPC-6, MS-GPC-8 and MS-GPC-10 identified thereby. The VH and VL families and the CDR3s listed refer to the HuCAL consensus-based antibody genes as described (Knappik et al., 2000); the sequences of the VH and VL CDRs are shown in 10 Table 1, and the full sequences of the VH and VL domains are shorn in Figure 15.

3. Generation of Fab-fragments

3.1. Conversion of scFv to Fab

The Fab-fragment antigen binding polypeptides MS-GPC-1-Fab, MS-GP-6-Fab, MS- 15 GPC-8-Fab and MS-GPC-10-Fab were generated from their corresponding scFv fragments as follows. Both heavy and light chain variable domains of scFv fragments were cloned into pMx9_Fab (Figure 13), the heavy chain variable domains as MfeI / StyI-fragments, the variable domains of the kappa light chains as EcoRV/ BsiWI-fragments. The lambda chains were first amplified from the corresponding 20 pMORPH13_scFv vector as template with PCR-primers CRT5 (5' primer) and CRT6 (3' primer), wherein CRT6 introduces a unique DraIII restriction endonuclease site.

CRT5: 5' GTGGTGGTTCCGATATC 3'

25 CRT6: 5' AGCGTCACACTCGGTGCGGCTTCGGCTGGCCAAGAACGGGTTA 3'

The PCR product is cut with EcoRV / DraIII and cloned into pMx9_Fab (see Figure 13). The Fab light chains could be detected with a polyclonal goat anti-human IgG 30 antibody-R-Phycoerythrin-conjugate (Jackson ImmunoResearch, West Grove, PA, USA, Cat. No. 109-116-088, F(ab')₂ fragment) (1:200 dilution).

3.2. Expression and purification of HuCAL-antibody fragments in E.coli

Expression in E.coli cells (JM83) of scFv and Fab fragments from pMx7_FS or pMx9_Fab, respectively, were carried out in one litre of 2 x TY-medium supplemented

with 34 µg/ml chloramphenicol. After induction with 0.5 mM IPTG (scFv) or 0.1 mM IPTG (Fab), cells were grown at 22°C for 12 hours. Cell pellets were lysed in a French Press (Thermo Spectronic, Rochester, NY, USA) in 20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole (pH 7.4). Cell debris was removed by centrifugation and 5 the clear supernatant filtered through 0.2 µm pores before subjecting it to STREP tag purification using a Streptactin matrix and purification conditions according to the supplier (IBA GmbH, Göttingen, Germany). Purification by size exclusion chromatography (SEC) was performed as described by Rheinnecker et al. (1996). The apparent molecular weights were determined by SEC with calibration standards 10 and confirmed in some instances by coupled liquid chromatography-mass spectrometry (TopLab GmbH, Martinsried, Germany).

4. Optimization of antibody fragments

In order to optimize certain biological characteristics of the HLA-DR binding antibody 15 fragments, one of the Fab fragments, MS-GPC-8-Fab, was used to construct a library of Fab antibody fragments by replacing the parental VL λ1 chain by the pool of all lambda chains λ 1-3 randomized in CDR3 from the HuCAL library (Knappik et al., 2000).

20 The Fab fragment MS-GPC-8-Fab (see 3.1) was cloned via XbaI/EcoRI from pMx9_Fab_GPC-8 into pMORPH18_Fab, a phagemid-based vector for phage display of Fab fragments, to generate pMORPH18_Fab_GPC-8 (see Figure 14). A lambda chain pool comprising a unique DraIII restriction endonuclease site (Knappik et al., 25 2000) was cloned into pMORPH18_Fab_GPC-8 cut with NsiI and DraIII (see vector map of pMORPH18_Fab_GPC-8 in Figure 14).

The resulting Fab optimization library was screened by two rounds of panning against 30 MHC-class II DRA*0101/DRB1*0401 (prepared as above) as described in 2.3 with the exception that in the second round the antigen concentration for coating was decreased to 12 ng/well. FACS identified optimized clones as described above in 2.5. Seven of these clones, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18 and MS-GPC-8-27, were further characterized and showed cell killing activity as found for the starting fragment MS-GPC-8. Table 1 contains the sequence characteristics of MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9,

MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18 and MS-GPC-8-27. The VH and VL families and the CDR3s listed refer to the HuCAL consensus-based antibody genes as described (Knappik et al., 2000). The full sequences of the VH and VL domains of MS-GPC-8-6, MS-GPC-8-10, MS-GPC-8-17 and MS-GPC-8-27 are shown in Figure 5 15.

The optimized Fab forms of the anti-HLA-DR antibody fragments MS-GPC-8-6 and MS-GPC-8-17 showed improved characteristics over the starting MS-GPC-8. For example, the EC₅₀ of the optimized antibodies was 15-20 and 5-20 nM (compared to 10 20-40 nM for MS-GPC-8, where the concentration is given as the concentration of the bivalent cross-linked Fab dimer), and the maximum capacity to kill MHH-Call 4 cells determined as 76 and 78% for MS-GPC-8-6 and MS-GPC-8-17 (compared to 65% for MS-GPC-8) respectively.

15 For further optimization, the VL CDR1 regions of a set of anti-HLA-DR antibody fragments derived from MS-GPC-8 (including MS-GPC-8-10 and MS-GPC-8-27) were optimized by cassette mutagenesis using trinucleotide-directed mutagenesis (Virnekäs et al., 1994). In brief, a VI1 CDR1 library cassette was synthesized containing six randomized positions (total variability: 7.43×10^6), and was cloned into 20 a VI1 framework. The CDR1 library was digested with EcoRV and BbsI, and the fragment comprising the CDR1 library ligated into the lambda light chains of the MS-GPC-8-derived Fab antibody fragments in pMORPH18_Fab (as described above), digested with EcoRV and BbsI. The resulting library was screened as described above. Ten clones were identified as above by binding specifically to HLA DR (MS- 25 GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 & MS-GPC- 30 8-27-41) and showed cell killing activity as found for the starting fragments MS-GPC-8, MS-GPC-8-10 and MS-GPC-8-27. Table 1 contains the sequence characteristics of MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 & MS-GPC-8-27-41. The VH and VL families and the CDR3s listed refer to the HuCAL consensus-based antibody genes as described (Knappik et al., 2000), the full sequences of the VH and VL domains of MS-GPC-8-6-13, MS-GPC-8-10-57 & MS-GPC-8-27-41 are shown in Figure 15.

From these 10 clones, four Fab fragments were chosen (MS-GPC-8-6-2, MS-GPC-8-6-13, MS-GPC-8-10-57 and MS-GPC-8-27-41) as demonstrating significantly improved EC50 of cell killing as described in example 10. Table 1 shows the sequences of clones optimised at the CDR1 region.

Optimisation procedures not only increased the biological efficacy of anti-HLA DR antibody fragments generated by the optimisation process, but a physical characteristic - affinity of the antibody fragment to HLA DR protein - was also substantially improved. For example, the affinity of Fab forms of MS-GPC-8 and its optimised descendants was measured using a surface plasmon resonance instrument (Biacore, Upsala Sweden) according to example 7. The affinity of the MS-GPC-8 parental Fab was improved over 100 fold from 346 nM to ~ 60 nM after VLCDR3 optimisation and further improved to single digit nanomolar affinity (range 3 – 9 nM) after VLCDR3+1 optimisation (Table 2).

5. Generation of IgG

5.1 Construction of HuCAL-immunoglobulin expression vectors

Heavy chains were cloned as follows. The multiple cloning site of pcDNA3.1+ (Invitrogen) was removed (NheI / ApaI), and a stuffer compatible with the restriction sites used for HuCAL-design was inserted for the ligation of the leader sequences (NheI / EcoRI), VH-domains (EcoRI / BpuI) and the immunoglobulin constant regions (BpuI / ApaI). The leader sequence (EMBL M83133) was equipped with a Kozak sequence (Kozak, 1987). The constant regions of human IgG1 (PIR J00228), IgG4 (EMBL K01316) and serum IgA1 (EMBL J00220) were dissected into overlapping oligonucleotides with lengths of about 70 bases. Silent mutations were introduced to remove restriction sites non-compatible with the HuCAL-design. The oligonucleotides were spliced by overlap extension-PCR.

Light chains were cloned as follows. The multiple cloning site of pcDNA3.1/Zeo+ (Invitrogen) was replaced by two different stuffers. The κ-stuffer provided restriction sites for insertion of a κ-leader (NheI / EcoRV), HuCAL-scFv Vκ-domains (EcoRV / BsiWI) and the κ-chain constant region (BsiWI / ApaI). The corresponding restriction

sites in the λ-stuffer were NheI / EcoRV (λ-leader), EcoRV / HpaI (Vλ- domains) and HpaI / Apal (λ-chain constant region). The κ-leader (EMBL Z00022) as well as the λ-leader (EMBL L27692) were both equipped with Kozak sequences. The constant regions of the human κ- (EMBL J00241) and λ -chain (EMBL M18645) were
5 assembled by overlap extension-PCR as described above.

5.2 Generation of IgG-expressing CHO-cells

All cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ in media recommended by the supplier. CHO-K1 (CRL-9618) were from ATCC and were co-

10 transfected with an equimolar mixture of IgG heavy and light chain expression vectors. Double-resistant transfectants were selected with 600 µg/ml G418 and 300 µg/ml Zeocin (Invitrogen) followed by limiting dilution. The supernatant of single clones was assessed for IgG expression by capture-ELISA. Positive clones were expanded in RPMI-1640 medium supplemented with 10% ultra-low IgG-FCS (Life
15 Technologies). After adjusting the pH of the supernatant to 8.0 and sterile filtration, the solution was subjected to standard protein A column chromatography (Poros 20A, PE Biosystems).

The IgG forms of anti-HLA-DR antigen binding domains show improved
20 characteristics over the antibody fragments. These improved characteristics include affinity (Example 7) and killing efficiency (Examples 9, 10 and 14).

6. HLA-DR specificity assay and epitope mapping

To demonstrate that antigen-binding domains selected from the HuCAL library bound
25 specifically to a binding site on the N-terminal domain of human MHCII receptor largely conserved between alleles and hitherto unknown in the context of cell killing by receptor cross linking, we undertook an assessment of their binding specificity, and it was attempted to characterise the binding epitope.

30 The Fab antibody fragments MS-GPC-8-27-7, MS-GPC-8-27-10, MS-GPC-8-6-13, MS-GPC-8-27-41, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-6-27, MS-GPC-8 and MS-GPC-8-6 showed specificity of binding to HLA-DR protein but not to non-HLA-DR proteins. Fab fragments selected from the HuCAL library were tested for reactivity with the following antigens: HLA-DR protein (DRA*0101/DRB1*0401;

prepared as example 1, and a set of unrelated non-HLA-DR proteins consisting of BSA, testosterone-BSA, lysozyme and human apotransferrin. An empty well (Plastic) was used as negative control. Coating of the antigen MHCII was performed over night at 1 µg/well in PBS (Nunc-MaxiSorp TM) whereas for the other antigens (BSA, 5 Testosterone-BSA, Lysozyme, Apotransferrin) 10 µg/well was used. Next day wells were blocked in 5% non-fat milk for 1 hr followed by incubation of the respective antibodies (anti-MHCII-Fabs and an unrelated Fab (Mac1-8A)) at 100 ng/well for 1h. After washing in PBS the anti-human IgG F(ab')2-peroxidase-conjugate at a 1:10000 dilution in TBS (supplemented with 5% w/v non-fat dry-milk/0.05% v/v Tween 20) was 10 added to each well for 1h. Final washes were carried out in PBS followed the addition the substrate POD (Roche). Color-development was read at 370 nM in an ELISA-Reader

All anti-HLA-DR antibody fragments MS-GPC-8-27-7, MS-GPC-8-27-10, MS-GPC-8-15 6-13, MS-GPC-8-27-41, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-6-27, MS-GPC-8 and MS-GPC-8-6 demonstrated high specificity for HLA-DR, as evidenced by the much higher mean fluorescence intensity resulting from incubation of these antibody fragments with HLA-DR derived antigens compared to controls (Figure 1a). In a similar experiment, the Fab fragments MS-GPC-1, MS-GPC-6, MS-GPC-8 and 20 MS-GPC-10 were found to bind to both the DRA*0101/DRB1*0401 (preparaed as above) as well as to a chimeric DR-IE consisting of the N-terminal domains of DRA*0101 and DRB1*0401 with the remaining molecule derived from a murine class II homologue IEd (Ito et al., 1996) (Figure 1b).

25 To demonstrate the broad-DR reactivity of anti-HLA-DR antibody fragments and IgGs of the invention, the scFv forms of MS-GPC-1, 6, 8 and 10, and IgG forms of MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-51 & MS-GPC-8-6-13 were tested for reactivity against a panel of Epstein-Barr virus transformed B cell lines obtained from ECACC (Salisbury UK), each homozygous for one of the most frequent DR alleles in 30 human populations (list of cell lines and alleles shown in Figure 2). The antibody fragments were also tested for reactivity against a series of L cells transfected to express human class II isotypes other than DRB1: L105.1, L257.6, L25.4, L256.12 & L21.3 that express the molecules DRB3*0101, DRB4*0101, DP0103/0402, DP 0202/0201, and DQ0201/0602 respectively (Klohe et al., 1988).

Reactivity of an antigen-binding fragment to the panel of cell-lines expressing various MHC- class II molecules was demonstrated using an immunofluorescence procedure as for example, described by Otten et al (1997). Staining was performed on 2×10^5 5 cells using an anti-FLAG M2 antibody as the second reagent against the M2 tag carried by each anti-HLA-DR antibody fragment and a fluorescein labelled goat anti-mouse Ig (BD Pharmingen, Torrey Pine, CA, USA) as a staining reagent. Cells were incubated at 4°C for 60 min with a concentration of 200 nM of the anti-HLA-DR antibody fragment, followed by the second and third antibody at concentrations 10 determined by the manufacturers. For the IgG form, the second antibody was omitted and the IgG detected using a FITC-labeled mouse anti-human IgG4 (Serotec, Oxford, UK) . Cells were washed between incubation steps. Finally the cells were washed and subjected to analysis using a FACS Calibur (BD Immunocytometry Systems, San Jose, CA, USA).

15

Figure 2 shows that the scFv-fragments MS-GPC-1, 6, 8 and 10, and IgG forms of MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-51 & MS-GPC-8-6-13 react with all DRB1 allotypes tested. This observation taken together with the observation that all 20 anti-HLA-DR antibody fragments react with chimeric DR-IE, suggests that all selected anti-HLA-DR antibody fragments recognize the extracellular first domain of the monomorphic DR α chain or a monomorphic epitope on extracellular first domain of the DR β chain.

We then attempted to localize the binding domains of MS-GPC-8-10-57 and MS- 25 GPC-8-27-41 further by examining competitive binding with murine antibodies for which the binding domains on HLA-DR are known. The murine antibodies L243 and LB3.1 are known to bind to the α 1 domain, 1-1C4 and 8D1 to the β 1 domain and 10F12 to the β 2 domain (Vidovic et al. 1995b). To this end, an assay was developed 30 wherein a DR-expressing cell line (LG-2) was at first incubated with the IgG4 forms of MS-GPC-8-10-57 or MS-GPC-8-27-41, the Fab form of MS-GPC-8-10-57 or the Fab form of GPC 8, and an unrelated control antibody. Subsequently murine antibodies were added and the murine antibodies were detected. If the binding site of MS-GPC- 8-10-57 or MS-GPC-8-27-41 overlaps with the binding of a murine antibody, then a reduced detection of the murine antibody is expected.

Binding of the IgG4 forms of GPC-8-27-41 and MS-GPC-8-10-57 and the Fab form of MS-GPC-8-10-57 substantially inhibited (mean fluorescence intensity reduced by > 90%) the binding of 1-1C4 and 8D1, whereas L243, LB3.1 and 10F12 and a control
5 were only marginally affected. The Fab form of MS-GPC-8 reduced binding of 1-1C4 by ~ 50% (mean fluorescence dropped from 244 to 118), abolished 8D1 binding and only marginally affected binding of L243, LB3.1 and 10F12 or the control. An unrelated control antibody had no effect on either binding. Thus, MS-GPC-8-10-57 and MS-GPC-8-27-41 seem to recognise a β 1 domain epitope that is highly
10 conserved among allelic HLA-DR molecules.

The whole staining procedure was performed on ice. 1×10^7 cells of the human B-lymphoblastoid cell line LG-2 was preblocked for 20 Min. in PBS containing 2% FCS and 35 μ g/ml Guinea Pig IgG ("FACS-Buffer"). These cells were divided into 3 equal
15 parts A, B, and C of approximately 3.3×10^6 cells each, and it was added to A.) 35 μ g MS-GPC-8-10-57 or MS-GPC-8-27-41 IgG4, to B.) 35 μ g MS-GPC-8-10-57 Fab or MS-GPC-8 Fab, and to C.) 35 μ g of an unrelated IgG4 antibody as negative control, respectively, and incubated for 90 min. Subsequently A, B, C were divided in 6 equal parts each containing 5.5×10^5 cells, and 2 μ g of the following murine antibodies were
20 added each to one vial and incubated for 30 min: 1.) purified mIgG ; 2.) L243; 3.) LB3.1; 4.) 1-1 C4; 5.) 8D1; 6.) 10F12. Subsequently, 4ml of PBS were added to each vial, the vials were centrifuged at 300g for 8 min, and the cell pellet resuspended in 50 μ l FACS buffer containing a 1 to 25 dilution of a goat-anti-murine Ig-FITC conjugate at 20 μ g/ml final concentration (BD Pharmingen, Torrey Pines, CA, USA). Cells were
25 incubated light-protected for 30 min. Afterwards, cells were washed with 4 ml PBS, centrifuged as above and resuspended in 500 μ l PBS for analysis in the flow cytometer (FACS Calibur, BD Immunocytometry Systems, San Jose, CA, USA).

The PepSpot technique (US 6040423; Heiskanen et al., 1999) is used to further
30 identify the binding epitope for MS-GPC 8-10-57. Briefly, an array of 73 overlapping 15mer peptides is synthesised on a cellulose membrane by a solid phase peptide synthesis spotting method (WO 00/12575). These peptide sequences are derived from the sequence of the α 1 and β 1 domains of HLA-DR4Dw14, HLA-DRA1*0101 (residues 1-81) and HLA-DRB1*0401 (residues 2-92), respectively, and overlap by

two amino acids. Second, such an array is soaked in 0.1% Tween-20/PBS (PBS-T), blocked with 5% BSA in PBS-T for 3 hours at room temperature and subsequently washed three times with PBS-T. Third, the prepared array is incubated for 90 minutes at room temperature with 50 ml of a 5 mg/l solution of the IgG form of GPC-8-10-57 in 5 1% BSA/PBS-T. Fourth, after binding, the membrane is washed three times with PBS-T and subsequently incubated for 1 hour at room temperature with a goat anti-human light chain antibody conjugated to horseradish peroxidase diluted 1/5000 in 1% BSA/PBS-T. Finally, the membrane is washed three times with PBS-T and any binding determined using chemiluminescence detection on X-ray film. As a control for 10 unspecific binding of the goat anti-human light chain antibody, the peptide array is stripped by the following separate washings each at room temperature for 30 min: PBS-T (2 times), water, DMF, water, an aqueous solution containing 8M urea, 1% SDS, 0.5% DTT, a solution of 50% ethanol, 10% acetic acid in water (3 times each) and, finally, methanol (2 times). The membrane is again blocked, washed, incubated 15 with goat anti-human I light chain antibody conjugated to horseradish peroxidase and developed as described above.

7. Affinity of anti- HLA-DR antibody and antibody fragments

In order to demonstrate the superior binding properties of anti-HLA antibody 20 fragments of the invention, we measured their binding affinities to the human MHC class II DR protein (DRA*0101/DRB1*0401) using standard equipment employing plasmon resonance principles. Surprisingly, we achieved affinities in the sub-nanomolar range for IgG forms of certain anti-HLA-DR antibody fragments of the invention. For example, the affinity of the IgG forms of MS-GPC-8-27-41, MS-GPC-8-25 6-13 & MS-GPC-8-10-57 was measured as 0.3, 0.5 and 0.6 nM respectively (Table 3a). Also, we observed high affinities in the range of 2-8 nM for Fab fragments affinity matured at the CDR1 and CDR3 light chain regions (Table 3b). Fab fragments affinity matured at only the CDR3 light chain region showed affinities in the range of 40 to 30 100 nM (Table 3c), and even Fab fragments of non-optimised HuCAL antigen binding domains showed affinities in the sub μ M range (Table 3d). Only a moderate increase in Kon (2-fold) was observed following CDR3 optimisation (Kon remained approximately constant throughout the antibody optimization process in the order of $1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$), whilst a significant decrease in Koff was a surprising feature of the optimisation process – sub 100 s^{-1} , sub 10 s^{-1} , sub 1 s^{-1} and sub 0.1 s^{-1} for the

unoptimised Fabs, CDR3 optimised Fabs, CDR3/CDR1 optimised Fabs and IgG forms of anti-HLA-DR antibody fragments of the invention.

The affinities for anti-HLA antibody fragments of the invention were measured as follows. All measurements were conducted in HBS buffer (20mM HEPES, 150mM NaCl, pH7.4) at a flow rate of 20µl/min at 25°C on a Biacore3000 instrument (Biacore AB, Sweden). MHC class II DR protein (prepared as example 1) was diluted in 100mM sodium acetate pH 4.5 to a concentration of 50 - 100 mg/ml, and coupled to a CM5 chip (Biacore AB) using standard EDC-NHS coupling chemistry with subsequent ethanolamine treatment as manufacturers directions. The coating density of MHCII was adjusted to between 500 and 4000 RU. Affinities were measured by injection of 5 different concentrations of the different antibodies and using the standard software of the Biacore instrument. Regeneration of the coupled surface was achieved using 10mM glycine pH2.3 and 7.5mM NaOH.

15

8. Multivalent killing activity of anti HLA-DR antibodies and antibody fragments

To demonstrate the effect of valency on cell killing, a cell killing assay was performed using monovalent, bivalent and multivalent compositions of anti-HLA-DR antibody fragments of the invention against GRANTA-519 cells. Anti-HLA-DR antibody fragments from the HuCAL library showed much higher cytotoxic activity when cross-linked to form a bivalent composition (60 – 90% killing at antibody fragment concentration of 200 nM) by co-incubation with anti-FLAG M2 mAb (Figure 3) compared to the monovalent form (5 – 30% killing at antibody fragment concentration of 200 nM). Incubation of cell lines alone or only in the presence of anti-FLAG M2 mAb without co-incubation of anti-HLA-DR antibody fragments did not lead to cytotoxicity as measured by cell viability. Treatment of cells as above but using 50 nM of the IgG4 forms (naturally bivalent) of the antibody fragments MS-GPC-8, MS-GPC-8-6-13, MS-GPC-8-10-57 and MS-GPC-8-27-41 without addition of anti-FLAG M2 mAb showed a killing efficiency after 4 hour incubation of 76%, 78%, 78% and 73% respectively.

Furthermore, we observed that higher order valences of the anti-HLA-DR antibody fragments further decrease cell viability significantly. On addition of Protein G to the incubation mix containing the IgG form of the anti-HLA-DR antibody fragments, the

multivalent complexes thus formed further decrease cell viability compared to the bivalent composition formed from incubation of the anti-HLA-DR antibody fragments with only the bivalent IgG form.

5 The killing efficiency of anti-HLA-DR antibody fragments selected from the HuCAL library was tested on the HLA-DR positive tumor cell line GRANTA-519 (DSMZ, Germany). 2×10^5 cells were incubated for 4 h at 37°C under 6% CO₂ with 200 nM anti-HLA-DR antibody fragments in RPMI 1640 (PAA, Germany) supplemented with 2,5% heat inactivated FBS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-
10 essential amino acids, 1 mM sodium pyruvate and 0,1 mg/ml kanamycin. Each anti-HLA-DR antibody fragment was tested for its ability to kill activated tumor cells as a monovalent anti-HLA-DR antibody fragment or as a bivalent composition by the addition of 100 nM of a bivalent cross-linking anti-FLAG M2 mAb. After 4 h incubation at 37°C under 6% CO₂, cell viability was determined by trypan blue staining and
15 subsequent counting of remaining viable cells (Current Protocols in Immunology, 1997).

The above experiment was repeated using KARPAS-422cells against a multivalent form of IgG forms of MS-GPC-8-10-57 and MS-GPC-8-27-41 prepared by a pre-
20 incubation with a dilution series of the bacterial protein Protein G. Protein G has a high affinity and two binding sites for IgG antibodies, effectively cross-linking them to yield a total binding valency of 4. In a control using IgG alone without preincubation with Protein G, approximately 55% of cells were killed, while cell killing using IgG pre-
incubated with Protein G gave a maximum of approximately 75% at a molar ratio of
25 IgG antibody/Protein G of ~ 6 (based on a molecular weight of Protein G of 28.5 kD). Higher or lower molar ratios of IgG antibody/Protein G approached the cell killing efficiency of the pure IgG antibodies.

9. Killing efficiency of anti-HLA-DR antibody fragments

30 Experiments to determine the killing efficiency of the anti-HLA-DR cross-linked antibody fragments against other tumor cell lines that express HLA-DR molecules were conducted analogous to example 8. Tumor cell lines that show greater than 50% cell killing with the cross linked Fab form of MS-GPC-8 after 4 h incubation include MHH-CALL4, MN 60, BJAB, BONNA-12 which represent the diseases B cell acute

lymphoid leukemia, B cell acute lymphoid leukemia, Burkitt lymphoma and hairy cell leukemia respectively. Use of the cross-linked Fab form of the anti-HLA-DR antibody fragments MS-GPC-1, 6 and 10 also shows similar cytotoxic activity to the above tumor cell lines when formed as a bivalent agent using the cross-linking anti-FLAG

5 M2 mAb.

The method described in example 8 was used to determine the maximum killing capacity for each of the cross-linked bivalent anti-HLA-DR antibody fragments against Priess cells. The maximum killing capacity observed for MS-GPC-1, MS-GPC-6, MS-

10 GPC-8 & MS-GPC-10 was measured as 83%, 88%, 84% and 88% respectively. Antibody fragments generated according to example 4, when cross linked using anti-FLAG M2 mAb as above, also showed improved killing ability against GRANTA and Priess cells (Table 4).

15 *10. Killing efficiency of anti-HLA-DR IgG antibodies of human composition*

Compared to corresponding murine antibodies (Vidovic et al, 1995b; Nagy & Vidovic, 1996; Vidovic & Toral; 1998), we were surprised to observe significantly improved killing efficiency of IgG forms of certain anti-HLA-DR antibody fragments of the invention (Table 5). Following the method described in examples 8 and 9 but at 50 nM, repeated measurements (3 to 5 replica experiments where cell number was counted in duplicate for each experiment) were made of the killing efficiency of the IgG forms of certain antibody fragments of the invention. When applied at a final concentration of only 50 nM, IgGs of the antibody fragments MS-GPC-8, MS-GPC-8-6-13, MS-GPC-8-10-57 & MS-GPC-8-27-41 killed more than 50% of cells from 16, 22, 25 19 and 20 respectively of a panel of 24 human tumor cell lines that express HLA-DR antigen at a level greater than 10 fluorescent units as determined by example 11. Cells were treated with the two murine anti-HLA-DR antibodies L243 (Vidovic et al, 1995b) and 8D1 (Vidovic & Toral; 1998) at a significantly higher final concentration of mAb (200 nM), which reduced cell viability to a level below 50% viable cells in only 13 30 and 12 of the 24 HLA-DR expressing cells lines, respectively. The cell line MHH-PREB-1 was singled out and not accounted as part of the panel of 24 cell lines despite its expression of HLA-DR antigen at a level greater than 10 fluorescent units due to the inability of any of the above antibodies to induce any significant reduction of cell viability. This is further explained in example 12.

Indeed, even at the significantly increased concentration, the two murine antibodies treated at 200 nM showed significantly less efficient killing compared to the IgG forms of anti-HLA DR antibody fragments of the invention. Not only do IgG forms of the
5 human anti-HLA-DR antibody fragments of the invention show an overall increase in cell killing at lower concentrations compared to the murine antibodies, but they show less variance in killing efficiency across different cell lines. The coefficient of variance in killing for the human antibodies in this example is 32% (mean %killing = 68 +/- 22% (SD)), compared to over 62% (mean %killing = 49 +/- 31% (SD)) for the mouse
10 antibodies. Statistically controlling for the effect on killing efficiency due to HLA expression by fitting logistic regression models to mean percentage killing against log(mean HLA DR expression) supports this observation (Figure 4). Not only is the fitted curve for the murine antibodies consistently lower than that for the human, but a larger variance in residuals from the murine antibody data (SD = 28%) is seen
15 compared to the variance in residuals from the human antibody data (16%).

11. Killing selectivity of antigen-binding domains against a human antigen for activated versus non-activated cells

Human peripheral B cells were used to demonstrate that human anti-HLA-DR mAb-
20 mediated cell killing is dependent on cell-activation. 50 ml of heparinised venous blood was taken from an HLA-DR typed healthy donor and fresh peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque Gradient Centrifugation (Histopaque-1077; Sigma) as described in Current Protocols in Immunology (John Wiley & Sons, Inc.; 1999). Purified B cells (~5% of peripheral blood leukocytes) were
25 obtained from around 5×10^7 PBMC using the B-cell isolation kit and MACS LS⁺/VS⁺ columns (Miltenyi Biotec, Germany) according to manufacturers guidelines. Successful depletion of non-B cells was verified by FACS analysis of an aliquot of isolated B cells (HLA-DR positive and CD19 positive). Double staining and analysis is done with commercially available antibodies (BD Immunocytometry Systems, San
30 Jose, CA, USA) using standard procedures as for example described in Current Protocols in Immunology (John Wiley & Sons, Inc.; 1999). An aliquot of the isolated B cells was tested for the ability of the cells to be activated by stimulation with Pokeweed mitogen (PWM) (Gibco BRL, Cat. No. 15360-019) diluted 1:25 in RPMI 1640 (PAA, Germany) supplemented with 10% FCS (Biowhittaker Europe, BE), 2mM

L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate and 0,1mg/ml kanamycin by incubation at 37°C under 6% CO₂ for three days. Successful activation was verified by FACS analysis of HLA-DR expression on the cell surface (Current Protocols in Immunology, John Wiley & Sons, Inc.; 1999).

5

The selectivity for killing of activated cells versus non-activated cells was demonstrated by incubating 1x10⁶/ml B cells activated as above compared to non-activated cells, respectively with 50 nM of the IgG forms of MS-GPC-8-10-57, MS-GPC-8-27-41 or the murine IgG 10F12 (Vidovic et al., 1995b) in the medium described above but supplemented with 2,5% heat inactivated FCS instead of 10%, or with medium alone. After incubation at 37°C under 6% CO₂ for 1 or 4h, cell viability was determined by fluorescein diacetate staining (FDA) of viable and propidium iodide staining (PI) of dead cells and subsequent counting of the green (FDA) and red (PI) fluorescent cells using a fluorescence microscope (Leica, Germany) using standard procedures (Current Protocols in Immunology, 1997).

B cell activation was shown to be necessary for cell killing. In non-activated cells after 1 h of incubation with the anti-HLA-DR antibodies, the number of viable cells in the media corresponded to 81%, 117% 126% and 96% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone, respectively. In contrast, the number of viable activated B cells after 1 h incubation corresponded to 23%, 42% 83% and 66% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone, respectively. After 4 h of incubation, 78%, 83% 95% and 97% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone were found viable in non-activated cells, whereas the cell density had dropped to 23%, 24% 53% and 67% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone, respectively, in activated cells.

30 *12. Killing activity of anti-HLA antibody fragments against the cell line MHH PreB 1*
As evidenced in Table 5, we observed that our cross-linked anti-HLA-DR antibody fragments or IgGs did not readily kill a particular tumor cell line expressing HLA-DR at significant levels. We hypothesized that although established as a stable cell line, cells in this culture were not sufficiently activated. Therefore, we conducted an

experiment to stimulate activity of the MHH preB1 cell line, using increased cell-surface expression of HLA-DR molecule as a marker of activation as follows.

Non-adherently growing MHH preB1 cells were cultivated in RPMI medium containing
5 the following additives (all from Gibco BRL and Bio Whittaker): 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 1x Kanamycin. Aliquots were activated to increase expression of HLA-DR molecule by incubation for one day with Lipopolysaccharide (LPS, 10 µg/ml), Interferon-gamma (IFN- γ , Roche, 40 ng/ml) and phyto-hemagglutinin (PHA, 5 µg/ml). The cell surface
10 expression of HLA-DR molecules was monitored by flow cytometry with the FITC-conjugated mAb L243 (BD Immunocytometry Systems, San Jose, CA, USA). Incubation of MHH preB1 for one day in the presence of LPS, IFN- γ and PHA resulted in a 2-fold increase in HLA-DR surface density (mean fluorescence shift from 190 to 390). Cell killing was performed for 4 h in the above medium but
15 containing a reduced FCS concentration (2.5%). A concentration series of the IgG forms of MS-GPC-8-27-41 & MS-GPC-8-10-57 was employed, consisting of final antibody concentrations of 3300, 550, 92, 15, 2.5, 0.42 and 0.07 nM, on each of an aliquot of non-activated and activated cells. Viable cells were identified microscopically by exclusion of Trypan blue. Whereas un-activated cell viability
20 remains unaffected by the antibody up to the highest antibody concentration used, cell viability is dramatically reduced with increasing antibody concentration in activated MHH PreB1 cells (Figure 5).

13. *Killing efficiency of anti-HLA-DR IgG antibodies of human composition against ex-vivo chronic lymphoid leukemia cells*
25

Using B cells isolated and purified from 10 patients suffering from chronic lymphoid leukemia (CLL), we demonstrated that IgG forms of anti-HLA-DR antibody fragments of the invention showed efficacy in killing of clinically relevant cells using an ex-vivo assay. B-cells were isolated and purified from 10 unrelated patients suffering from
30 CLL (samples kindly provided by Prof Hallek, Ludwig Maximillian University, Munich) according to standard procedures (Buhmann et al., (1999)). 2×10^5 cells were treated with 100 nM of IgG forms of the anti-HLA-DR antibody fragments MS-GPC-8, MS-GPC-8-10-57 or MS-GPC-8-27-41 and incubated for 4 or 24 hours analogous to examples 8 and 9. A replica set of cell cultures was established and activated by

incubation with HeLa-cells expressing CD40 ligand on their surface for three days before treatment with antibody (Buhmann et al., 1999). As controls, the murine IgG 10F12 (Vidovic et al., 1995b) or no antibody was used. Cell viability for each experiment was determined as described in example 12.

5

Surprisingly, IgG forms of the anti-HLA-DR antibody fragments of the invention showed highly efficient and uniform killing - even across this diverse set of patient material. After only 4 hours of treatment, all three human IgGs gave a significant reduction in cell viability compared to the controls, and after 24 hours only 33% of 10 cells remained viability (Figure 6). We found that on stimulating the ex-vivo cells further according to Buhmann et al (1999), the rate of killing was increased such that after only 4 hours culture with the human antibodies, only 24% of cells remained viable on average for all patient samples and antibody fragments of the invention.

15 **14. Determination of EC50 for anti-HLA-DR antibody fragments**

We demonstrated superior Effective Concentration at 50% effect (EC50) values in a cell-killing assay for certain forms of anti-HLA-DR antibody fragments selected from the HuCAL library compared to cytotoxic murine anti-HLA-DR antibodies (Table 6).

20 The EC50 for anti-HLA-DR antibody fragments selected from the HuCAL library were estimated using the HLA-DR positive cell line PRIESS or LG2 (ECACC, Salisbury UK). 2×10^5 cells were incubated for 4 h at 37°C under 6% CO₂ in RPMI 1640 (PAA, Germany) supplemented with 2,5% heat inactivated FBS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate and 25 0,1mg/ml kanamycin, together with dilution series of bivalent anti-HLA-DR antibody fragments. For the dilution series of Fab antibody fragments, an appropriate concentration of Fab fragment and anti-FLAG M2 antibody were premixed to generate bivalent compositions of the anti-HLA-DR antibody fragments. The concentrations stated refer to the concentration of bivalent composition such that the 30 IgG and Fab EC50 values can be compared.

After 4 h incubation with bivalent antibody fragments at 37°C under 6% CO₂, cell viability was determined by fluorescein diacetate staining and subsequent counting of remaining viable cells (Current Protocols in Immunology, 1997). Using standard

statistical software, non-linear logistic regression curves were fitted to replica data points and the EC50 estimated for each antibody fragment.

When cross-linked using the anti-FLAG M2 antibody, the Fab fragments MS-GPC-1,
5 MS-GPC-8 & MS-GPC-10 selected from the HuCAL library (Example 4) showed an
EC50 of less than 120 nM as expressed in terms of the concentration of the
monovalent fragments, which corresponds to a 60 nM EC50 for the bivalent cross-
linked (Fab)dimer-anti-Flag M2 conjugate. (Figure 7a). When cross-linked using the
anti-FLAG M2 antibody, anti-HLA-DR antibody fragments optimised for affinity within
10 the CDR3 region (Example 4) showed a further improved EC50 of less than 50 nM, or
25 nM in terms of the bivalent cross-linked fragment (Figure 7b), and those
additionally optimised for affinity within the CDR1 region showed an EC50 of less than
30 nM (15 nM for bivalent fragment). In comparison, the EC50 of the cytotoxic murine
15 anti-HLA-DR antibodies 8D1 (Vidovic & Toral; 1998) and L243 (Vidovic et al; 1995b)
showed an EC50 of over 30 and 40 nM, respectively, within the same assay (Figure
7c).

Surprisingly, the IgG form of certain antibody fragments of the invention showed
approximately 1.5 orders of magnitude improvement in EC50 compared to the murine
20 antibodies (Figure 7d). For example, the IgG forms of MS-GPC-8-10-57 & MS-GPC-
8-27-41 showed an EC50 of 1.2 and 1.2 nM respectively. Furthermore, despite being
un-optimised for affinity, the IgG form of MS-GPC-8 showed an EC50 of less than 10
nM.

25 As has been shown in examples 11 and 12, the efficiency of killing of un-activated
cells (normal peripheral B and MHH PreB cells respectively) is very low. After
treatment with 50 nM of the IgG forms of MS-GPC-8-10-57 & MS-GPC-8-27-41, 78%
and 83% of normal peripheral B cells, respectively, remain viable after 4 hours.
Furthermore, at only 50nM concentration or either IgG, virtually 100% viability is seen
30 for MHH PreB1 cells. Indeed, a decrease in the level of viability to below 50% cannot
be achieved with these un-activated cells using reasonable concentration ranges (0.1
to 300 nM) of IgG or bivalent cross-linked Fab forms of the anti-HLA DR antibody
fragments of the invention. Therefore, the EC50 for these un-activated cell types can
be estimated to be at least 5 times higher than that shown for the non-optimised Fab

forms (EC50 ~ 60 nM with respect to cross-linked bivalent fragment), and at least 10 times and 100 times higher than EC50s shown for the VHCDR3 optimised Fabs (~ 25 nM with respect to cross-linked bivalent fragment) and IgG forms of MS-GPC-8-10-57 (~1.2 nM) & MS-GPC-8-27-41 (~1.2 nM) respectively.

5

15. Mechanism of cell-killing

The examples described above show that cell death occurs - needing only certain multivalent anti-HLA-DR antibody fragments to cause killing of activated cells. No further cytotoxic entities or immunological mechanisms were needed to cause cell 10 death, therefore demonstrating that cell death is mediated through an innate pre-programmed mechanism of the activated cell. The mechanism of apoptosis is a widely understood process of pre-programmed cell death. We were surprised by certain characteristics of the cell killing we observed that suggested the mechanism of killing for activated cells when exposed to our human anti-HLA-DR antibody 15 fragments was not what is commonly understood in the art as "apoptosis". For example, the observed rate of cell killing appeared to be significantly greater than the rate reported for apoptosis of immune cells (about 10 - 15 h; Truman et al., 1994). Two experiments were conducted to demonstrate that the mechanism of cell killing proceeded by a non-apoptotic mechanism.

20

First, we used Annexin-V-FITC and propidium iodide (PI) staining techniques to distinguish between apoptotic and non-apoptotic cell death – cells undergoing apoptosis, "apoptotic cells", (Annexin-V positive/PI negative) can be distinguished from necrotic ("Dead") (Annexin-V positive/PI positive) and fully functional cells 25 (Annexin-V negative/PI negative). Using the procedures recommended by the manufacturers of the AnnexinV and PI assays, 1×10^6 /ml Priess cells were incubated at 37°C under 6% CO₂ with or without 200 nM anti-HLA-DR antibody fragment MS-GPC-8 together with 100 nM of the cross-linking anti-FLAG M2 mAb in RPMI 1640 (PAA, DE) supplemented with 2,5% heat inactivated FCS (Biowhittaker Europe, BE), 30 2mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 0,1 mg/ml kanamycin. To provide an apoptotic cell culture as control, 1×10^6 /ml Priess cells were induced to enter apoptosis by incubation in the above medium at 37°C under 6% CO₂ with 50 µg/ml of the apoptosis-inducing anti-CD95 mAb DX2 (BD Pharmingen, Torrey Pine, CA, USA) cross-linked with 10 µg/ml Protein-G. At various

incubation times (1, 15 and 60 min, 3 and 5 h) 200 µl samples were taken, washed twice and stained with Annexin-V-FITC (BD Pharmingen, Torrey Pine, CA, USA) and PI using Annexin-V binding buffer following the manufacturer's protocol. The amount of staining with Annexin-V-FITC and PI for each group of cells is analysed with a
5 FACS Calibur (BD Immunocytometry Systems, San Jose, CA, USA).

Cell death induced through the cross-linked anti-HLA-DR antibody fragments shows a significantly different pattern of cell death than that of the anti-CD95 apoptosis inducing antibody or the cell culture incubated with anti-FLAG M2 mAb alone.
10 The percentage of dead cells (as measured by Annexin-V positive/PI positive staining) for the anti-HLA-DR antibody fragment/anti-FLAG M2 mAb treated cells increases far more rapidly than that of the anti-CD95 or the control cells (Figure 8a). In contrast, the percentage of apoptotic cells (as measured by Annexin-V positive/PI negative staining) increases more rapidly for the anti-CD95 treated cells compared to the
15 cross-linked anti-HLA-DR antibody fragments or the control cells (Figure 8b).

Second, we inhibited caspase activity using zDEVD-fmk, an irreversible Caspase-3 inhibitor, and zVAD-fmk, a broad spectrum Caspase inhibitor (both obtained from BioRad, Munich, DE). The mechanism of apoptosis is characterized by activity of
20 caspases, and we hypothesized that if caspases were not necessary for anti HLA-DR mediated cell death, we would observe no change in the viability of cells undergoing cell death in the presence of these caspase inhibitors compared to those without.
25 2×10^5 Priess cells were preincubated for 3 h at 37°C under 6% CO₂ with serial dilutions of the two caspase inhibitors ranging from 180 µM to 10 mM in RPMI 1640 (PAA, DE) supplemented with 2,5% heat inactivated FCS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate and 0,1mg/ml kanamycin. HLA-DR mediated cell death was induced by adding 200 nM of the human anti-HLA-DR antibody fragment MS-GPC-8 and 100 nM of the cross-linking anti-M2 mAb. An anti-CD95 induced apoptotic cell culture served as a control
30 for the activity of inhibitors (Drenou et al., 1999). After further incubation at 37°C and 6% CO₂, cell viability after 4 and 24 h was determined by trypan blue staining and subsequent counting of non-stained cells. As we expected, cell viability of the anti-HLA-DR treated cell culture was not significantly modified by the presence of the Caspase inhibitors, while cell death induced through anti-CD95 treatment was

significantly decreased for the cell culture pre-incubated with the Caspase inhibitors. This observation supports our hypothesis that HLA-DR mediated cell death proceeds through a non-apoptotic mechanism that is independent of caspase proteases that can be inhibited by zDEVD-fm or zVAD-fmk.

5

16. In vivo therapy for cancer using an HLA-DR specific antibody

We demonstrate that antigen-binding domains of human composition can successfully be used as a therapeutic for the treatment of cancer.

Immunocompromised mice - such as scid, nude or Rag-1 knockout - are inoculated

10 with a DR+ human lymphoma or leukemia cell line of interest. The tumor cell dose, usually 1×10^6 to 1×10^7 /mouse, is established for each tumor tested and administered

subcutaneously (s.c.) or intravenously (i.v.). The mice are treated i.v. or s.c with the IgG form of the anti-HLA-DR antibody fragments MS-GPC-8, MS-GPC-8-10-57, MS-

GPC-8-27-41 or others of the invention prepared as described above, using doses of

15 1 to 25 mg/kg over 5 days. Survival of anti-HLA-DR treated and control untreated mice is monitored for up to 8 weeks after cessation of treatment. Tumor progression

in the mice inoculated s.c. is additionally quantified by measuring tumor surface area.

Significant prolongation of survival of up to 80% of anti-HLA-DR treated mice is observed during the experiment, and up to 50% mice survive at the end of the

20 experiment. In s.c. inoculated and untreated mice, the tumor reaches a surface area of 2 - 3 cm², while in anti-HLA-DR treated animals the tumor surface area is significantly less.

17. Immunosuppression using anti-HLA-DR antibody fragments measured by

25 *reduction in IL-2 secretion*

We were surprised to observe that certain anti-HLA DR antibody fragments of the invention displayed substantial immunomodulatory properties within an assay measuring IL-2 secretion from immortalized T-cells. IgG forms of the antibody

30 fragments MS-GPC-8-6-13, MS-GPC-8-10-57 & MS-GPC-8-27-41 showed very strong immunosuppressive properties in this assay with sub-nanomolar IC50 values

and virtually 100% maximal inhibition (Figure 9a). Particularly surprising was our observation that certain monovalent compositions of the antibody fragments of the invention were able to strongly inhibit IL-2 secretion in the same assay. For example, Fab forms of the VHCDR3-selected and VLCDR3/VLCDR1 optimised antibody

fragments showed low single-digit nano-M IC₅₀s and also almost 100% maximal inhibition (Figure 9b). Other monovalent anti-HLA DR antibody fragments of the invention showed significant immunosuppressive properties in the assay compared to control IgG and Fab fragments (Table 7).

5

The immunomodulatory properties of anti-HLA DR antibody fragments was investigated by measuring IL-2 secretion from the hybridoma cell line T-Hyb 1 stimulated using DR-transgenic antigen presenting cells (APC) under conditions of half-maximal antigen stimulation. IL-2 secretion was detected and measured using a

10 standard ELISA method provided by the OptiEIA mouse IL-2 kit of Pharmingen (Torrey Pine, CA, USA). APCs were isolated from the spleen of unimmunized chimeric 0401-IE transgenic mice (Ito et al. 1996) according to standard procedures.

15 1.5×10^5 APCs were added to 0.2 ml wells of 96-well in RPMI medium containing the following additives (all from Gibco BRL and PAA): 10 % FCS, 2mM L-glutamine, 1%

non-essential amino acids, 1 mM sodium pyruvate and 0.1 g/l kanamycin. Hen egg 20 ovalbumin was added to a final concentration of 200 µg/ml in a final volume of 100 µl of the above medium, the cells incubated with this antigen for 30 min at 37°C under 6% CO₂. Anti-HLA DR antibody fragments were added to each well at various concentrations (typically in a range from 0.1 to 200 nM), the plate incubated for 1 h at

25 37°C/6% CO₂ and 2×10^5 T-Hyb 1 cells added to give a final volume of 200 µl in the above medium. After incubation for 24 h, 100 µl of supernatant was transferred to an ELISA plate (Nunc-Immuno Plate MaxiSorp surface, Nunc, Roskilde, DK) previously coated with IL-2 Capture Antibody (BD Pharmingen, Torrey Pine, CA, USA), the amount of IL-2 was quantified according to the manufacturer's directions using the

25 OptiEIA Mouse IL-2 kit and the plate read using a Victor V reader (Wallac, Finland). Secreted IL-2 in pg/ml was calibrated using the IL-2 standards provided in the kit.

The T-cell hybridoma line T-Hyb1 was established by fusion of a T-cell receptor negative variant of the thymoma line BW 5147 (ATCC) and lymph node cells from

30 chimeric 0401-IE transgenic mice previously immunized with hen egg ovalbumin (Ito et al. 1996). The clone T-Hyb1 was selected for the assay since it responded to antigen specific stimulation with high IL-2 secretion.

18. Immunosuppression using an HLA-DR specific antibody measured by T cell proliferation

Immunomodulatory properties of the anti-HLA DR antibody fragments were also seen within an assay that measures T cell proliferation. The IC₅₀ value for inhibition of T cell proliferation of the IgG form of MS-GPC-8-10-57 and MS-GPC-8-27-41 were 11 and 20 nM respectively (Figure 10). The anti-HLA DR antibody fragments were tested as follows to inhibit the proliferative T cell response of antigen-primed lymph node cells from mice carrying a chimeric mouse-human class II transgene with an RA-associated peptide binding site, and lack murine class II molecules (Muller et al., 10 1990; Woods et al., 1994; Current Protocols in Immunology, Vol. 2, 7.21; Ito et al., 1996). Here, the immunization takes place *in vivo*, but the inhibition and readout are *ex vivo*. Transgenic mice expressing MHC class II molecules with binding sites of the RA associated molecule, DRB*0401 were commercially obtained. These mice lack murine MHC class II, and thus, all Th responses are channelled through a single 15 human RA-associated MHC class II molecule (Ito et al. 1996). These transgenic mice represent a model for testing human class II antagonists.

The inhibitory effect of the anti-HLA-DR antibody fragments and their IgG forms were tested on T-cell proliferation measured using chimeric T-cells and antigen presenting 20 cells isolated from the lymph nodes of chimeric 0401-IE transgenic mice (Taconic, USA) previously immunized with hen egg ovalbumin (Ito et al. 1996) according to standard procedures. 1.5×10^5 cells are incubated in 0.2 ml wells of 96-well tissue culture plates in the presence of ovalbumin (30 µg per well - half-maximal stimulatory concentration) and a dilution series of the anti-HLA DR antibody fragment or IgG form 25 under test (0.1 nM - 200 nM) in serum free HL-1 medium containing 2 mM L-glutamine and 0.1 g/l Kanamycin for three days. Antigen specific proliferation is measured by ³H-methyl-thymidin(1 µCi/well) incorporation during the last 16h of culture (Falcioni et al., 1999). Cells are harvested, and ³H incorporation measured 30 using a scintillation counter (TopCount, Wallac Finland). Inhibition of T-cell proliferation on treatment with the anti-HLA DR antibody fragment and its IgG form may be observed by comparison to control wells containing antigen.

19. Selection of useful polypeptide for the treatment of cancers

In order to select the most appropriate protein/peptide to enter further experiments and to assess its suitability for use in a therapeutic composition for the treatment of cancers, additional data are collected. Such data for each IgG form of the anti-HLA

5 antigen antibody fragments can include the binding affinity, *in vitro* killing efficiency as measured by EC50 and cytotoxicity across a panel of tumor cell lines, the maximal percentage cell killing as estimated *in vitro*, and tumor reduction data and mouse survival data from *in vivo* animal models.

10 The IgG form of the anti-HLA antigen antibody fragments that shows the highest affinity, the lowest EC50 for killing, the highest maximal percentage cell killing and broadest across various tumor cell lines, the best tumor reduction data and/or the best mouse-survival data may be chosen to enter further experiments. Such experiments may include, for example, therapeutic profiling and toxicology in animals
15 and phase I clinical trials in humans.

20. Selection of useful polypeptide for the treatment of diseases of the immune system

In order to select the most appropriate protein/peptide to enter further experiments

20 and to assess its suitability for use in a therapeutic composition for the treatment of diseases of the immune system, additional data are collected. Such data for each monovalent antibody fragment or IgG form of the anti-HLA antigen antibody fragments can include the affinity, reactivity, specificity, IC50-values, for inhibition of IL-2 secretion and of T-cell proliferation, or *in vitro* killing efficiency as measured by
25 EC50 and the maximal percentage cell killing as estimated *in vitro*, and DR-transgenic models of transplant rejection and graft vs. host disease.

The antibody fragment or IgG form of the anti-HLA antigen antibody fragments that shows the lowest EC50, highest affinity, highest killing, best specificity and/or greatest
30 inhibition of T-cell proliferation or IL-2 secretion, and high efficacy in inhibiting transplant rejection and/or graft vs. host disease in appropriate models, might be chosen to enter further experiments. Such experiments may include, for example, therapeutic profiling and toxicology in animals and phase I clinical trials in humans.

Table 1:

VH and VL families, VL CDR1 and VH/VL CDR 3 sequences of HLA-DR-specific polypeptides

Clone	VH CDR3 Length	VH-CDR3-Seq.	VL	VL-CDR1-Seq.	CDR3 Length	VL-CDR3-Seq.	Families
MS-GPC-1	H2 10	QYGHRGGFDH	λ 1	SGSSNIGSNYVS	8	QSYDFNES	H2 λ 1
MS-GPC-6	H3 9	GYGRYSPDL	K3	RASQQSVSSSYLA	8	QQYSNLPF	H3 K 3
MS-GPC-8	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDMPQA	H2 λ 1
MS-GPC-10	H2 10	QLHYRGGFDL	λ 1	SGSSSNIGSNYVS	8	QSYDLTMG	H2 λ 1
MS-GPC-8-1	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDFSHY	H2 λ 1
MS-GPC-8-6	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDYDHY	H2 λ 1
MS-GPC-8-9	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDIQLH	H2 λ 1
MS-GPC-8-10	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDLIRH	H2 λ 1
MS-GPC-8-17	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDFSVY	H2 λ 1
MS-GPC-8-18	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDFSIY	H2 λ 1
MS-GPC-8-27	H2 10	SPRYRGAFDY	λ 1	SGSSSNIGSNYVS	8	QSYDMNVH	H2 λ 1

MS-GPC-8-6-2	H2	10	SPRYRGAFDY	λ 1	SGSESNIGSNYVH	8	QSYDYDHY	H2 λ 1
MS-GPC-8-6-19	H2	10	SPRYRGAFDY	λ 1	SGSESNIGSNYVA	8	QSYDYDHY	H2 λ 1
MS-GPC-8-6-27	H2	10	SPRYRGAFDY	λ 1	SGSDSNIGANYVT	8	QSYDYDHY	H2 λ 1
MS-GPC-8-6-45	H2	10	SPRYRGAFDY	λ 1	SGSEPNIGSNYVF	8	QSYDYDHY	H2 λ 1
MS-GPC-8-6-13	H2	10	SPRYRGAFDY	λ 1	SGSESNIGANYVT	8	QSYDYDHY	H2 λ 1
MS-GPC-8-6-47	H2	10	SPRYRGAFDY	λ 1	SGSESNIGSNYVS	8	QSYDYDHY	H2 λ 1
MS-GPC-8-10-57	H2	10	SPRYRGAFDY	λ 1	SGSESNIGNNYVQ	8	QSYDLIRH	H2 λ 1
MS-GPC-8-27-7	H2	10	SPRYRGAFDY	λ 1	SGSESNIGNNYVG	8	QSYDMNVH	H2 λ 1
MS-GPC-8-27-10	H2	10	SPRYRGAFDY	λ 1	SGSESNIGANYVN	8	QSYDMNVH	H2 λ 1
MS-GPC-8-27-41	H2	10	SPRYRGAFDY	λ 1	SGSESNIGNNYVQ	8	QSYDMNVH	H2 λ 1

Table 2:

Steps in Antibody optimisation	Fab	$k_{on} [\text{s}^{-1}\text{M}^{-1}] \times 10^5$ +/- SD	$k_{off} [\text{s}^{-1}] \times 10^{-3}$ +/- SD	$K_D [\text{nM}]$ +/- SD	L-CDR3	L-CDR1
Parental Fab	MS-GPC-8	0.99 ± 0.40	29.0 ± 8.40	346.1 ± 140.5 ^{a)}	QSYDMMPQA	SGSSSNIGSNYVS
L-CDR3-optim.	-8-1	1.93	20.9	108 ^{e)}		
L-CDR3-optim.	-8-6	0.96 ± 0.14	5.48 ± 0.73	58.6 ± 11.7 ^{b)}		
L-CDR3-optim.	-8-9	1.85	16.6	90.1 ^{e)}		
L-CDR3-optim.	-8-10	nd	7.0 ^{e)}	nd		
L-CDR3-optim.	-8-17	1.0	5.48	54.7 ^{e)}		
L-CDR3-optim.	-8-18	1.06	8.3	78.3 ^{e)}		
L-CDR3-optim.	-8-27	nd	6.6 ^{e)}	nd		
L-CDR3-optim.	-8-6	0.96 ± 0.14	5.48 ± 0.73	58.6 ± 11.7 ^{b)}	QSYDYDHY	SGSSSNIGSNYVS
L-CDR3+1-opt.	-8-6-2	1.23 ± 0.11	0.94 ± 0.07	7.61 ± 0.25 ^{c)}	QSYDYDHY	SGSESNIGSNYVH
L-CDR3+1-opt.	-8-6-19	1.10 ± 0.08	0.96 ± 0.15	8.74 ± 1.33 ^{c)}	QSYDYDHY	SGSESNIGSNYVA
L-CDR3+1-opt.	-8-6-27	1.80 ± 0.24	1.10 ± 0.15	6.30 ± 0.63 ^{d)}	QSYDYDHY	SGSDSNIGANYVT
L-CDR3+1-opt.	-8-6-45	1.20 ± 0.07	1.03 ± 0.04	8.63 ± 0.61 ^{c)}	QSYDYDHY	SGSEPNIGSNYVF
L-CDR3+1-opt.	-8-6-13	1.90 ± 0.26	0.55 ± 0.05	2.96 ± 0.46 ^{c)}	QSYDYDHY	SGSESNIGANYVT
L-CDR3+1-opt.	-8-6-47	1.97 ± 0.29	0.62 ± 0.04	3.18 ± 0.33 ^{c)}	QSYDYDHY	SGSESNIGSNYVS
L-CDR3+1-opt.	-8-10-57	1.65 ± 0.21	0.44 ± 0.06	2.67 ± 0.25 ^{c)}	QSYDLIRH	SGSESNIGNNNYVQ
L-CDR3+1-opt.	-8-27-7	1.74 ± 0.21	0.57 ± 0.07	3.30 ± 0.34 ^{d)}	QSYDMNVH	SGSESNIGNNNYVG
L-CDR3+1-opt.	-8-27-10	1.76 ± 0.21	0.53 ± 0.05	3.01 ± 0.21 ^{c)}	QSYDMNVH	SGSESNIGANYVN

L-CDR3+1-opt.	-8-27-41	1.67 ± 0.16	0.49 ± 0.03	2.93 ± 0.27 ^{d)}	QSYDMNVH	SGSESNIGNNNYVQ
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- a) Affinity data of MS-GPC-8 are based on 8 different Fab-preparations which were measured on 4 different chips (2 x 500, 1000, 1000, 4000RU)
 - b) For MS-GPC-8-6 mean and standard deviation of 3 different preparations on 3 different chips (500, 4000, 3000RU) is shown.
 - c) 3000RU MHCII were immobilized on a CM5-chip. For each measurement 7 different concentrations from 1μM to 16nM were injected on the surface. Dissociation time: 150sec, regeneration was reached by 6μl 10mM Glycine pH2.3 followed by 8μl 7.5mM NaOH. For MS-GPC-8-6-19 mean and standard deviation of 4 different preparations are shown whereas for all other binders mean and standard deviation of 3 different preparations are shown.
 - d) One protein preparation is measured on 3 different chips (3000, 2800 and 6500RU).
 - e) Affinity determination of matured MHCII binder on a 4000RU density chips; single measurement.
- Molecular weights were determined after size exclusion chromatography and found 100% monomeric with the right molecular weight between 45 and 48 kDa.

Table 3a

Affinities of selected IgG4 monoclonal antibodies constructed from F_{ab}'s. Errors represent standard deviations

Binder (IgG ₄)	k _{on} [M ⁻¹ s ⁻¹] × 10 ⁵	k _{off} [s ⁻¹] × 10 ⁻⁵	K _D [nM]
MS-GPC-8-27-41	1.1 ± 0.2	3,1 ± 0.4	0,31 ± 0.06
MS-GPC-8-6-13	0,7 ± 0.1	3 ± 1	0,5 ± 0.2
MS-GPC-8-10-57	0,7 ± 0.2	4 ± 1	0,6 ± 0.2

Table 3b

Affinities of binders obtained out of affinity maturation of CDR1 light chain optimisation following CDR3 heavy chain optimisation. Errors represent standard deviations

Binder (F _{ab})	k _{on} [M ⁻¹ s ⁻¹] × 10 ⁵	k _{off} [s ⁻¹] × 10 ⁻³	K _D [nM]
MS-GPC-8-6-2	1.2 ± 0.1	0.94 ± 0.07	7.6 ± 0.3
MS-GPC-8-6-19	1.1 ± 0.1	1.0 ± 0.2	9 ± 1
MS-GPC-8-6-27	1.8 ± 0.2	1.1 ± 0.2	6.3 ± 0.6
MS-GPC-8-6-45	1.20 ± 0.07	1.03 ± 0.04	8.6 ± 0.6
MS-GPC-8-6-13	1.9 ± 0.3	0.55 ± 0.05	3.0 ± 0.5
MS-GPC-8-6-47	2.0 ± 0.3	0.62 ± 0.04	3.2 ± 0.3
MS-GPC-8-10-57	1.7 ± 0.2	0.44 ± 0.06	2.7 ± 0.3
MS-GPC-8-27-7	1.7 ± 0.2	0.57 ± 0.07	3.3 ± 0.3
MS-GPC-8-27-10	1.8 ± 0.2	0.53 ± 0.05	3.0 ± 0.2
MS-GPC-8-27-41	1.7 ± 0.2	0.49 ± 0.03	2.9 ± 0.3

Table 3c

Binders obtained out of affinity maturation of GPC8 by CDR3 light chain optimisation

Binder (F_{ab})	$k_{on} [M^{-1}s^{-1}] \times 10^5$	$k_{off} [s^{-1}] \times 10^{-3}$	$K_D [nM]$
MS-GPC 8-18	1.06	8.3	78.3
MS-GPC 8-9	1.85	16.6	90.1
MS-GPC 8-1	1.93	20.9	108
MS-GPC 8-17	1.0	5.48	54.7
MS-GPC-8-6 ^{a)}	1.2 +/- 0.1	5.5 +/- 0.7	8 +/- 12

Chip density 4000RU MHCII

a) For MS-GPC-8-6 mean and standard deviation of 3 different preparations on 3 different chips (500, 4000, 3000RU) is shown.

Table 3d

Binders obtained out of HuCAL in scFv form and their converted Fabs

Binder	scFv			F_{ab}		
	$k_{on} [M^{-1}s^{-1}] \times 10^5$	$k_{off} [s^{-1}] \times 10^{-3}$	$K_D [nM]$	$k_{on} [M^{-1}s^{-1}] \times 10^5$	$k_{off} [s^{-1}] \times 10^{-3}$	$K_D [nM]$
MS-GPC 1	0.413	61	1500	0.639	53	820
MS-GPC 6	0.435	200	4600	0.135	114	8470 (1 curve)
MS-GPC 8	0.114	76	560	0.99 +/- 0.40	29.0 +/- 8.4	346 ^{a)} +/- 141
MS-GPC 10	0.187	180	9625	0.22	63	2860

Chip density 500RU MHCII

a) Affinity data of MS-GPC-8 are based on 8 different Fab-preparations which were measured on 4 different chips (2 x 500, 1000, 4000RU) and are shown with standard deviation.

Table 4

Killing efficiency after 4 hour incubation of cells with cross-linked anti-HLA-DR antibody fragments, and maximum killing after 24 hour incubation

Cross-linked Fab fragment	Killing efficiency against GRANTA	Maximum killing against Priess
MS-GPC-1	+	+
MS-GPC-6	+	+
MS-GPC-8	+	+
MS-GPC-10	+	+
MS-GPC-8-6	++	++
MS-GPC-8-17	++	++
MS-GPC-8-6-13	+++	+++
MS-GPC-8-10-57	+++	+++
MS-GPC-8-27-41	+++	+++

Table 5

Killing efficiency of anti-HLA-DR IgG antibodies of human composition compared to murine anti-HLA-DR antibodies against a panel of lymphoid tumor cell lines.

Name	DR type	Type	L243	mean-FL	% Killing by mAb			human mAbs		
					L243	8D1	MS-GPC-8	8-27-41	8-10-57	8-6-13
LG-2	1,1	B-lymphoblastoid	458	79	85	86	87	88	88	82
Priess	4,4	B-lymphoblastoid	621	87	83	85	88	93	93	74
ARH-77	12	B-lymphoblastoid	301	88	73	84	85	88	88	87
GRANTA-519	2,11	B cell non-Hodgkin	1465	83	56	76	78	78	78	73
KARPAS-422	2,4	B cell non-Hodgkin	186	25	32	51	66	68	68	71
KARPAS-299	1,2	T cell non-Hodgkin	919	78	25	81	82	79	79	76
DOHH-2	1,2	B cell lymphoma	444	29	23	58	59	60	60	53
SR-786	1,2	T cell lymphoma	142	3	8	1	53	44	44	26
MHH-CALL-4	1,2	B-ALL	348	35	41	43	63	46	46	43
MN-60	10,13	B-ALL	1120	46	22	71	69	66	66	67
BJAB	12,13	Burkitt lymph.	338	53	59	49	71	67	67	64
RAJI	10, 17	Burkitt lymph.	617	69	64	81	84	86	86	83
L-428	12	Hodgkin's lymph.	244	82	81	82	91	91	91	92

HDLM-2	Hodgkin's lymph.	326	77	73	89	88	84	90
HD-MY-Z	Hodgkin's lymph.	79	35	39	49	69	57	72
KM-H2	Hodgkin's lymph.	619	81	56	75	86	88	87
L1236	Hodgkin's lymph.	41	52	62	44	63	66	66
BONNA-12	hairy cell leuk.	2431	92	91	91	92	91	86
HC-1	hairy cell leuk.	372	88	89	89	93	86	93
NALM-1	CML	1078	44	4	83	82	78	65
L-363	plasma cell leu.	49	6	5	26	26	24	19
EOL-1	AML (eosinophil)	536	22	13	36	69	49	53
LP-1	multiple myeloma	315	12	0	61	73	70	73
RPMI-8226	multiple myeloma	19	6	0	14	29	26	19
MHH-PREB-1	B cell non-Hodgkin	175	3	3	3	2	4	8
MHH-CALL-2	B cell precursor leu.	+	5	5	5	5	5	5
OPM-2	multiple myeloma	3	13	0	8	1	4	5
KASUMI-1	AML	5	0	0	8	10	10	6
HL-60	AML	3	18	0	3	15	9	22
LAMA-84	CML	7	7	9	5	11	5	7

% Killing: 100 - % viable cells after a 4h treatment with 200 nM murine or 50 nM human mAb at 37°C.

Table 6

EC50 values for certain anti-HLA-DR antibody fragments of the invention in a cell-killing assay against lymphoid tumor cells. All EC50 refer to nanomolar concentrations of the bivalent agent (IgG or cross-linked Fab) such that values for cross-linked Fab and IgG forms can be compared.

Antibody fragment	Form	Cell line tested	EC50 of cell killing (nM) +/- SE for bivalent agent
MS-GPC-1	Fab	PRIESS	54 ± 14
MS-GPC-8	Fab	PRIESS	31 ± 9
MS-GPC-10	Fab	PRIESS	33 ± 5
MS-GPC-8-17	Fab	PRIESS	16 ± 4
MS-GPC-8-6-2	Fab	PRIESS	8 ± 2
MS-GPC-8-10-57	Fab	LG2	7.2
MS-GPC-8-27-41	Fab	LG2	7.2
MS-GPC-8-27-41	Fab	PRIESS	7.7
MS-GPC-8	IgG4	PRIESS	8.3
MS-GPC-8-27-41	IgG4	PRIESS	1.1 ± 0.1
MS-GPC-8-10-57	IgG4	PRIESS	1.1 ± 0.2
MS-GPC-8-27-41	IgG4	LG2	1.23 ± 0.2
MS-GPC-8-10-57	IgG4	LG2	1.0 ± 0.1
8D1	mIgG	PRIESS	33
L243	mIgG	PRIESS	47

Table 7

IC50 values for certain anti-HLA-DR antibody fragments of the invention in an assay to determine IL-2 secretion after antigen-specific stimulation of T-Hyb 1 cells. IC50 for the IgG forms (bivalent) are represented as molar concentrations, while in order to provide easy comparison, IC50s for the Fab forms (monovalent) are expressed in terms of half the concentration of the Fab to enable direct comparison to IgG forms.

Anti-HLA-DR antibody fragment	Form	IC50 (IgG/nM) ((Fab)/2/nM)		Maximum inhibition(%)
		Mean	SE	
MS-GPC-8-10-57	IgG	0.31	0.01	100
MS-GPC-8-27-41	IgG	0.28	0.07	100
MS-GPC-8-6-13	IgG	0.42	0.06	100
MS-GPC-8-6-2	IgG	3.6	1.1	100
MS-GPC-8-6	IgG	6.7	2.0	100
MS-GPC-8	IgG	11.0	0.8	100
MS-GPC-8-6-2	Fab	4.7	1.9	100
MS-GPC-8-6-13	Fab	2.1	0.8	100
MS-GPC-8-6-19	Fab	5.3	0.2	100
MS-GPC-8-10-57	Fab	2.9	1.0	100
MS-GPC-8-6-27	Fab	3.0	1.2	100
MS-GPC-8-6-47	Fab	2.6	0.6	100
MS-GPC-8-27-7	Fab	5.9	2.2	100
MS-GPC-8-27-10	Fab	7.3	1.9	100
MS-GPC-8-27-41	Fab	3.6	0.7	100
MS-GPC-8-6	Fab	20		100
MS-GPC-8	Fab	110		100

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Claims

1. A composition including a polypeptide comprising an antibody-based antigen-binding domain of human composition with binding specificity for an antigen expressed on the surface of a human cell, wherein treating cells expressing said antigen with a multivalent polypeptide having two or more of said antigen-binding domains causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing.
2. A composition including a polypeptide comprising an antibody-based antigen-binding domain which binds to human HLA DR with a K_d of 1 μ M or less, wherein treating cells expressing HLA DR with a multivalent polypeptide having two or more of said antigen-binding domains causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing.
3. A composition including a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition which specifically bind to human HLA DR, wherein treating cells expressing HLA DR with said multivalent polypeptide causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing, wherein said antigen-binding domains individually bind to human HLA DR with a K_d of 1 μ M or less.
4. A composition including a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition which specifically bind to human HLA DR, wherein treating cells expressing HLA DR with said multivalent polypeptide causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said cell killing, wherein said multivalent polypeptide has an EC₅₀ of 100 nM or less for killing activated lymphoid cells.
5. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain that binds to human HLA DR with a K_d of 1 μ M or less, said antigen-binding domain being isolated by a method which includes isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to at least one epitope of human HLA DR, wherein treating cells expressing HLA DR with a multivalent polypeptide having two or more of said antigen binding domains causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing.

6. The composition of claim 5, wherein the method for isolating the antigen-binding domain includes the further steps of:
 - a. generating a library of variants of at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
 - b. isolation of VL and VH domains from the library of variants by ability to bind to human HLA DR with a K_d of 1 μ M or less.
7. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ for killing transformed cells at least 5-fold lower than the EC₅₀ for killing normal cells.
8. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ for killing activated cells at least 5-fold lower than the EC₅₀ for killing unactivated cells.
9. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ of 50nM or less for killing transformed cells.
10. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ for killing lymphoid tumor cells of 10nM or less.
11. The composition of any of claim 1-6 or 8, wherein the multivalent polypeptide kills activated lymphoid cells.
12. The composition of claim 11, wherein said activated lymphoid cells are lymphoid tumor cells representing a disease selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, and multiple myeloid leukemia.
13. The composition of claim 11, wherein said activated lymphoid cells are from a cell line taken from the list of Priess, GRANTA-519, KARPAS-422, KARPAS-299, DOHH-2, SR-786, MHH-CALL-4, MN-60, BJAB, RAJI, L-428, HDLM-2, HD-MY-Z, KM-H2, L1236, BONNA-12, HC-1, NALM-1, L-363, EOL-1, LP-1, RPMI-8226, and MHH-PREB-1 cell lines.
14. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ of 100nM or less for killing cells of at least one of lymphoid tumor cell lines selected from the list of KARPAS-422, DOHH-2, SR-7, MHH-CALL-4, MN-60, HD-MY-Z, NALM-1 and LP-1.

15. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ of 50nM or less for killing cells from at least one lymphoid tumor cell line selected from the list of KARPAS-422, DOHH-2, MN-60, NALM-1 and LP-1.
16. The composition of any of claims 1-6, wherein the multivalent polypeptide has an EC₅₀ of 10nM or less for killing cells from at least one B cell lymphoblastoid cell line selected from the list LG2 and Priess.
17. The composition of any of claims 1-6, wherein said cells are non-lymphoid cells that express MHC class II molecules
18. The composition of any of claims 1-6, wherein said antigen-binding domain binds to the β-chain of HLA-DR.
19. The composition of claim 18, wherein said antigen-binding domain binds to the first domain of the β-chain of HLA-DR.
20. The composition of any of claims 1-6, wherein said antigen-binding domain binds to one or more HLA-DR types selected from the group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402, DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRw53-B4*0101 and DRw52-B3*0101.
21. The composition of claim 20, wherein said antigen-binding domain binds to at least 5 different of said HLA-DR types.
22. The composition of any one of claims 1-6, wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.
23. The composition of any one of claims 1-6, wherein said antigen-binding domain includes a combination of HuCAL VH2 and HuCAL VΛ1, wherein the VH CDR3, VL CDR1 And VL CDR3 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

24. The composition of any one of claims 1-23, wherein said antigen-binding domain includes a combination of HuCAL VH2 and HuCAL VL1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and/or

wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

wherein each n independently represents any amino acid residue.

25. The composition of claim 24, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

26. The composition of any one of claims 1-23, wherein said antigen-binding domain competes for antigen binding with an antibody including a combination of HuCAL VH2 and HuCAL VL1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

each n independently represents any amino acid residue; and/or

the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

each n independently represents any amino acid residue.

27. The composition of claim 26, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

28. The composition of any one of claims 1-27, wherein said antigen-binding domain includes a VL CDR1 sequence represented in the general formula

SGSnnNIGnNYVn

wherein each n independently represents any amino acid residue.

29. The composition of claim 28, wherein the CDR1 sequence is SGSESNIGNNYVQ.

30. The composition of any of claims 1-29, wherein the mechanism of said killing involves an innate pre-programmed process of said cell.
31. The composition of claim 30, wherein said killing is non-apoptotic.
32. The composition of claim 30, wherein said killing is dependent on the action of non-caspase proteases, and/or wherein said killing cannot be inhibited by zVAD-fmk or zDEVD-fmk.
33. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide including at least a F(ab')₂ antibody fragment or a mini-antibody fragment.
34. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least two monovalent antibody fragments selected from Fv, scFv, dsFv and Fab fragments, and further comprises a cross-linking moiety or moieties.
35. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least one full antibody selected from the antibodies of classes IgG1, 2a, 2b, 3, 4, IgA, and IgM.
36. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide that is formed prior to binding to a cell.
37. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide that is formed after binding to a cell.
38. The composition of claim 3 or 4, wherein the antigen binding sites are cross-linked to a polymer.
39. A nucleic acid comprising a protein coding sequence for an antigen-binding domain comprised in any of claims 1-32, or a multivalent polypeptide thereof.
40. A vector comprising the nucleic acid of claim 39, and a transcriptional regulatory sequence operably linked thereto.
41. A host cell harboring at least one nucleic acid of claim 39 or the vector of claim 40.
42. A method for the production of composition comprising a multivalent polypeptide that causes or leads to killing of cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing, comprising culturing the cells

of claim 41 under conditions wherein the nucleic acid is expressed either as a multivalent polypeptide or as a polypeptide comprising at least one antigen binding domains which is subsequently treated to form a multivalent polypeptide composition.

43. The composition of any of claims 1-38, formulated in a pharmaceutically acceptable carrier and/or diluent.
44. The use of a composition of any of claims 1-38, for preparing a pharmaceutical preparation for the treatment of animals.
45. The use of a nucleic acid of claim 39 for preparing a pharmaceutical preparation for the treatment of animals
46. The use of a host cell of claim 41 for preparing a pharmaceutical preparation for the treatment of animals
47. The use of the method of claim 42 for preparing a pharmaceutical preparation for the treatment of animals
48. The use according to claim 44-47, wherein said animal is a human.
49. The use according to claim 44-48, for the treatment of cell proliferative disorders, wherein said antibody-based antigen binding domain is part of a multivalent polypeptide.
50. The use according to claim 49, wherein said treatment is the treatment of disorders involving transformed cells expressing MHC class II antigens.
51. The use according to claim 49 or 50, wherein said treatment is the treatment of a disorder selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, and multiple myeloid leukemia.
52. The use according to any of claims 44-48, wherein said treatment is the treatment of disorders involving unwanted activation of cells of the immune system, such as lymphoid cells expressing MHC class II.
53. The use according to any of claims 44-48, wherein said treatment is the treatment of a disorder selected from rheumatoid arthritis, juvenile arthritis, multiple sclerosis, Grave's disease, insulin-dependent diabetes, narcolepsy, psoriasis, systemic lupus erythematosus, ankylosing spondylitis, transplant rejection, graft vs. host disease, Hashimoto's disease, myasthenia gravis, pemphigus vulgaris, glomerulonephritis, thyroiditis, pancreatitis, insulitis, primary biliary cirrhosis, irritable bowel disease and Sjogren syndrome.

54. The use according to any of claims 44-48, wherein said disorder is selected from myasthenia gravis, rheumatoid arthritis, multiple sclerosis, transplant rejection and graft vs. host disease.
55. A diagnostic composition including the composition of any of claims 1-38.
56. A diagnostic composition including the composition of any of claims 1-38 and a cross-linking moiety or moieties.
57. A method for killing a cell expressing an antigen on the surface of said cell comprising the step of treating the cell with a plurality of antigen-binding domains of any one of claims 1-38, wherein said antibody-based antigen-binding domains are part of a multivalent polypeptide, and where neither cytotoxic entities nor immunological mechanisms are needed to causes or leads to said killing
58. A method to identify patients that can be treated with a composition of any of claims 1-38, formulated in a pharmaceutically acceptable carrier and/or diluent comprising the steps of
 - a. Isolating cells from a patient;
 - b. Contacting said cells with the composition of any of claims 1-38; and
 - c. Measuring the degree of killing or immunosuppression of said cells.
59. A kit to identify patients that can be treated with a composition of any of claims 1-38, formulated in a pharmaceutically acceptable carrier and/or diluent comprising
 - a. A composition of any of claims 1-38; and
 - b. Means to measure the degree of killing or immunosuppression of said cells.
60. A kit comprising
 - a. a composition according to any one of claims 1-38, and
 - b. a cross-linking moiety.
61. A kit comprising
 - a. a composition according to any one of claims 1-38, and
 - b. a detectable moiety or moieties, and
 - c. reagents and/or solutions to effect and/or detect binding of (i) to an antigen.
62. A cytotoxic composition comprising a composition of any one of claims 1-38 operably linked to a cytotoxic agent.

63. An immunogenic composition comprising a composition of any one of claims 1-38 operably linked to an immunogenic agent.
64. A method to kill a cell comprising contacting said cell with a composition of any one of claims 1-38 operably linked a cytotoxic or immunogenic agent.
65. The use of a composition of any one of claims 1-38 operable linked a cytotoxic or immunogenic agent for preparing a pharmaceutical preparation for the treatment of animals.
66. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a K_d of 1 μ M or less, wherein treating cells expressing said antigen with said polypeptide causes or leads to suppression of an immune response.
67. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for human HLA DR antigen, wherein treating cells expressing HLA DR with said polypeptide causes or leads to suppression of an immune response, and wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.
68. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a K_d of 1 μ M or less, said antigen-binding domain being isolated by a method which includes isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to human MHC class II antigen, wherein treating cells expressing MHC Class II with said polypeptide causes or leads to suppression of an immune response.
69. The composition of claim 68, wherein the method for isolating the antigen-binding domain includes the further steps of:
 - a. generating a library of variants at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
 - b. isolation of VL and VH domains from the library of variants by ability to bind to human MHC class II antigen with a K_d of 1 μ M or less;

- c. (optionally) repeating steps (a) and (b) with at least one other of the CDR1, CDR2 and CDR3 sequences.
70. The composition of any of claims 67, 68 or 69, wherein said antigen-binding domain binds to HLA-DR
71. The composition of any of claims 66 or 70 wherein said antigen-binding domain binds to the β -chain of HLA-DR.
72. The composition of claim 71, wherein said antigen-binding domain binds to an epitope of the first domain of the β -chain of HLA-DR.
73. The composition of any of claims 66-72, wherein said cells are lymphoid cells.
74. The composition of any of claims 66-72, wherein said cells are non-lymphoid cells and express MHC class II antigens.
75. The composition of any of claims 66-74, having an IC₅₀ for suppressing an immune response of 1 μ M or less.
76. The composition of any of claims 66-74, having an IC₅₀ for inhibition of IL-2 secretion of 1 μ M or less
77. The composition of any of claims 66-74, having an IC₅₀ for inhibiting T cell proliferation of 1 μ M or less
78. The composition of any of claims 66-77, wherein said antigen-binding domain binds to one or more HLA-DR types selected from the group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402, DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRw53-B4*0101 and DRw52-B3*0101.
79. The composition of claim 78, wherein said antigen-binding domain binds to at least 5 different of said HLA-DR types.
80. The composition of any of claims 66-79, wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

81. The composition of any one of claims 66-77, wherein said antigen-binding domain includes of a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3, VL CDR1 And VL CDR3 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.
82. The composition of any of claims 66-77, wherein said antigen-binding domain includes a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and/or
wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

wherein each n independently represents any amino acid residue.

83. The composition of claim 82, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.
84. The composition of any of claims 66-77, wherein said antigen-binding domain competes for antigen binding with an antibody including a combination of HuCAL VH2 and HuCAL V λ 1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

each n independently represents any amino acid residue; and/or
the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

each n independently represents any amino acid residue.

85. The composition of claim 84, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.
86. The composition of any of claims 66-85, wherein said antigen-binding domain includes a VL CDR1 sequence represented in the general formula

SGSnnNIGnNYVn

- wherein each n independently represents any amino acid residue.
87. The composition of claim 86, wherein the CDR1 sequence is SGSESNIGNNYVQ.
 88. The composition of any one of claims 66-85, wherein said suppression of an immune response is brought about by or manifests itself in down-regulation of expression of said antigen expressed on the surface of said cell.
 89. The composition of any one of claims 66-85, wherein said suppression of an immune response is brought about by or manifests itself in inhibition of the interaction between said cell and other cells, wherein said interaction would normally lead to an immune response.
 90. The composition of any one of claims 66-85, wherein said suppression of the immune response is brought about by or manifests itself in the killing of said cells.
 91. The composition of claim 90, wherein said killing is mediated by binding of a plurality of antigen-binding domains, wherein said antibody-based antigen-binding domains are part of a multivalent polypeptide, and where neither cytotoxic entities nor immunological mechanisms are needed to causes or leads to said killing.
 92. The composition of any one of claims 66-91, formulated in a pharmaceutically acceptable carrier and/or diluent
 93. A pharmaceutical preparation comprising the composition of claim 75 in an amount sufficient to suppress an immune response in an animal.
 94. A pharmaceutical preparation comprising the composition of claim 76 in an amount sufficient to inhibit IL-2 secretion in an animal.
 95. A pharmaceutical preparation comprising the composition of claim 77 in an amount sufficient to inhibit T cell proliferation in an animal.
 96. The use of a composition of any one of claims 66-91, for preparing a pharmaceutical preparation for the treatment of animals, such as where said animals are human.
 97. A nucleic acid including a protein coding sequence for a polypeptide of the composition of any of claims 66-91.
 98. A vector comprising the coding sequence of claim 97, and a transcriptional regulatory sequence operably linked thereto.
 99. A host cell harboring a nucleic acid of claim 97 or the vector of claim 98.
 100. A method for the production of an immunosuppressive composition, comprising culturing the cells of claim 99 under conditions wherein the nucleic acid is expressed.

101. A method for suppressing activation of a cell of the immune system, such as expressing HLA DR, comprising treating the cell with a composition of any of claims 66-92.
102. A method for suppressing proliferation of a cell of the immune system, such as expressing HLA DR, comprising treating the cell with a composition of any of claims 66-92.
103. A method for suppressing IL-2 secretion by a cell of the immune system, such as expressing HLA DR, comprising treating the cell with a composition of any of claims 66-92
104. A method for immunosuppressing a patient, comprising administering to the patient an effective amount of a composition of any of claims 66-92 to reduce the level of immunological responsiveness in the patient.
105. A method for killing a cell expressing an antigen on the surface of said cell comprising the step of treating the cell with a plurality of antigen-binding domains of any one of claims 66-87, wherein said antibody-based antigen-binding domains are part of a multivalent polypeptide, and where neither cytotoxic entities nor immunological mechanisms are needed to causes or leads to said killing, such where said antigen is HLA DR.
106. The use according to claim 96, wherein said treatment is the treatment of a disorder selected from rheumatoid arthritis, juvenile arthritis, multiple sclerosis, Grave's disease, insulin-dependent diabetes, narcolepsy, psoriasis, systemic lupus erythematosus, ankylosing spondylitis, transplant rejection, graft vs. host disease, Hashimoto's disease, myasthenia gravis, pemphigus vulgaris, glomerulonephritis, thyroiditis, pancreatitis, insulitis, primary biliary cirrhosis, irritable bowel disease and Sjogren syndrome.
107. The use according to claim 96, wherein said treatment is the treatment of a disorder selected from myasthenia gravis, rheumatoid arthritis, multiple sclerosis, transplant rejection and graft vs. host disease.
108. A method of suppressing the interaction of a cell of the immune system with an other cell, comprising contacting the cell with the composition of any of claims 66-92.
109. A method for conducting a pharmaceutical business comprising:
 - (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;

- (ii) generating a multivalent composition, such as multivalent polypeptide, comprising a plurality of said antigen-binding domains, which multivalent composition kills with an EC₅₀ of 50nM or less transformed or activated cells that express said antigen, where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing.;
 - (iii) conducting therapeutic profiling of the multivalent composition, for efficacy and toxicity in animals;
 - (iv) preparing a package insert describing the multivalent composition for treatment of proliferative disorders; and
 - (v) marketing the multivalent composition for treatment of proliferative disorders.
110. A method for conducting a life science business comprising:
- (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;
 - (ii) generating a multivalent composition, such as multivalent polypeptide, comprising a plurality of said antigen-binding domains, which multivalent composition kills with an EC₅₀ of 50nM or less transformed or activated cells expressing said antigen where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing;
 - (iii) licensing, jointly developing or selling, to a third party, the rights for selling the multivalent composition.
111. The method of any of claims 109 or 110, wherein the antigen-binding domain is isolated by a method which includes
- a. isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to HLA DR,
 - b. generating a library of variants at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
 - c. isolation of VL and VH domains from the library of variants by ability bind to HLA DR with a K_d of 1μM or less.
112. A method for conducting a pharmaceutical business comprising:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a K_d of 1 μ M or less;
- (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an IC_{50} of 100nM or less;
- (iii) conducting therapeutic profiling of the composition for efficacy and toxicity in animals;
- (iv) preparing a package insert describing the use of the composition for immunosuppression therapy; and
- (v) marketing the composition for use as an immunosuppressant.

113. A method for conducting a life science business comprising:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a K_d of 1 μ M or less;
- (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an IC_{50} of 100nM or less;
- (iii) licensing, jointly developing or selling, to a third party, the rights for selling the composition.

114. The method of any of claims 112 or 113, wherein the antigen-binding domain is isolated by a method which includes

- a. isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to HLA DR,
- b. generating a library of variants at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
- c. isolation of VL and VH domains from the library of variants by ability to bind to HLA DR with a K_d of 1 μ M or less.

115. The method of any of claims 109-114, wherein said antigen-binding domain comprises a combination of VH and VL domains found in the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

Figure 1a

	MS-GPC- 8-27-7	MS-GPC- 8-27-10	MS-GPC- 8-6-13	MS-GPC- 8-27-41	MS-GPC- 8-6-47	MS-GPC- 8-10-57	MS-GPC- 8-6-27	MS-GPC- 8	MS-GPC- 8-6
Plastic	-0,004	-0,02	-0,022	-0,025	-0,001	0,005	0,007	-0,022	-0,018
BSA	-0,003	-0,019	-0,021	-0,022	0,008	0,003	0,003	-0,016	-0,019
Testosterone	-0,005	-0,01	-0,012	-0,007	0,011	0,003	0,002	-0,009	-0,012
-BSA									
Lysozyme	-0,005	-0,079	-0,079	-0,073	0,013	0,014	0,006	-0,081	-0,072
human Apotransferrin	-0,009	-0,016	-0,018	-0,018	-0,005	-0,008	-0,004	-0,014	-0,016
MHCII (DRA*0101/ DRB1*0401)	1,549	1,493	1,467	1,525	1,4	1,256	1,297	1,058	1,306

Figure 1b

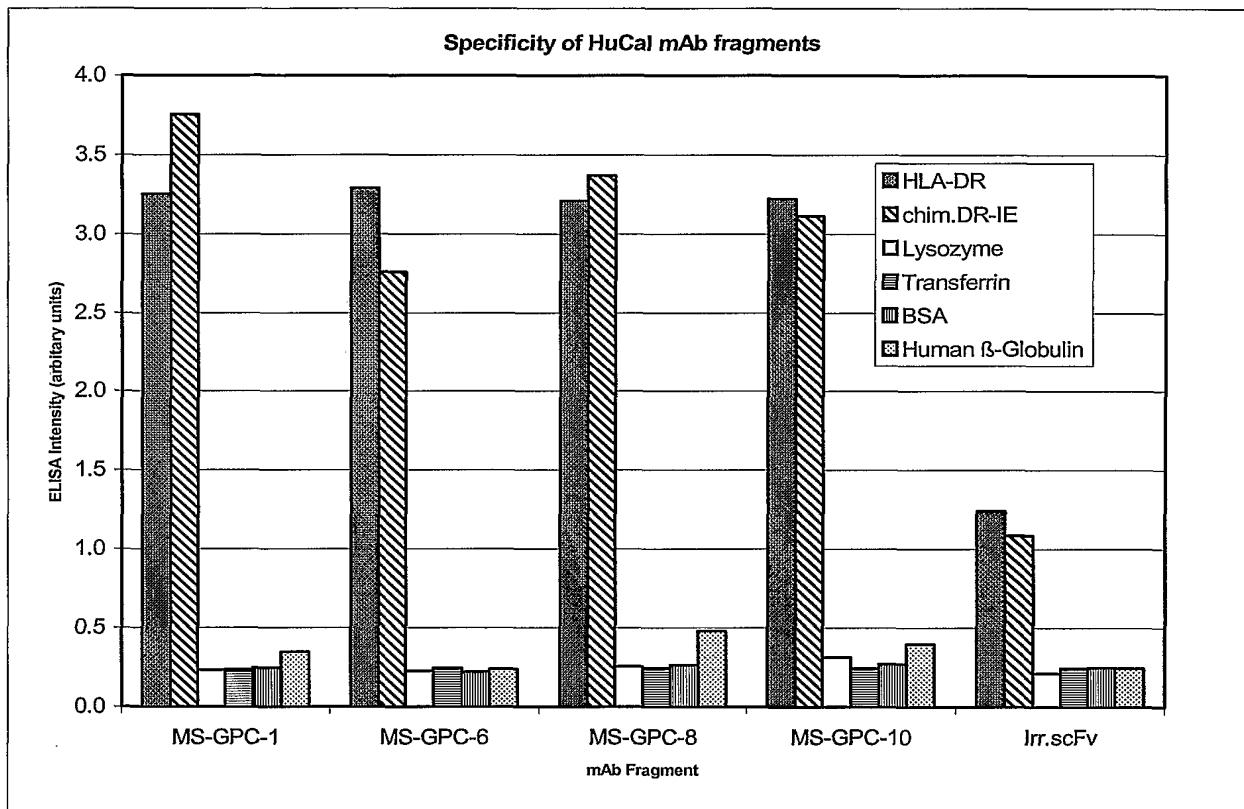


Figure 2

Cell line	HLA-	DRB1*	ScFv				IgG				
			MS- GPC- 1	MS- GPC- 6	MS- GPC- 8	MS- GPC- 10	MS- GPC- 8	MS- GPC- 8	MS- GPC- 8-10-57	MS- GPC- 8-27-41	MS- GPC- 8-6-13
LG2	DR1	0101	+	+	+	+	+	+	+	+	+
E4181324	DR2	15021	+	+	+	+	+	+	+	+	+
VAVY	DR3	0301	+	+	+	+	+	+	+	+	+
Priess	DR4Dw4	0401	+	+	+	+	+	+	+	+	+
TS10	DR4Dw10	0402	+	+	+	+	+	+	+	+	+
BIN40	DR4Dw14	0404	+	+	+	+	+	+	+	+	+
WT47	DR6	1302	+	+	+	+	+	+	+	+	+
TEM	DR6	1401	+	+	+	+	+	+	+	+	+
TAB089	DR8	8031	+	+	+	+	+	+	+	+	+
DKB	DR9	9012	+	+	+	+	+	+	+	+	+
L257.6	DRw53	B4*0101	+/-	+	+	+/-	nt	nt	nt	nt	nt
L105.1	DRw52	B3*0101	+	+	+	+	nt	nt	nt	nt	nt
L25.4	DPw4/w4.2	DP0103/0402	-	+	-	-	nt	nt	nt	nt	nt
L256.12	DPw2/w2.1	DP0202/0201	-	+/-	-	-	nt	nt	nt	nt	nt
L21.3	DQ7/w2	DQ0201/0602	-	+	+	-	nt	nt	nt	nt	nt

Figure 3

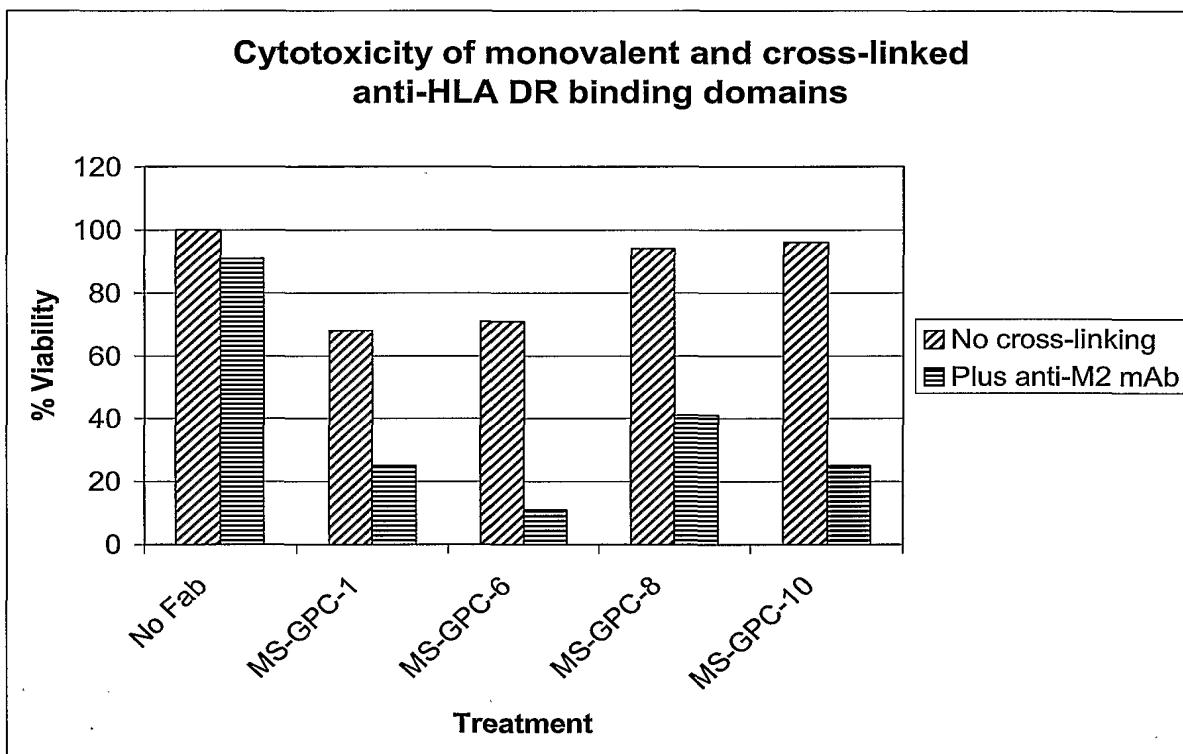


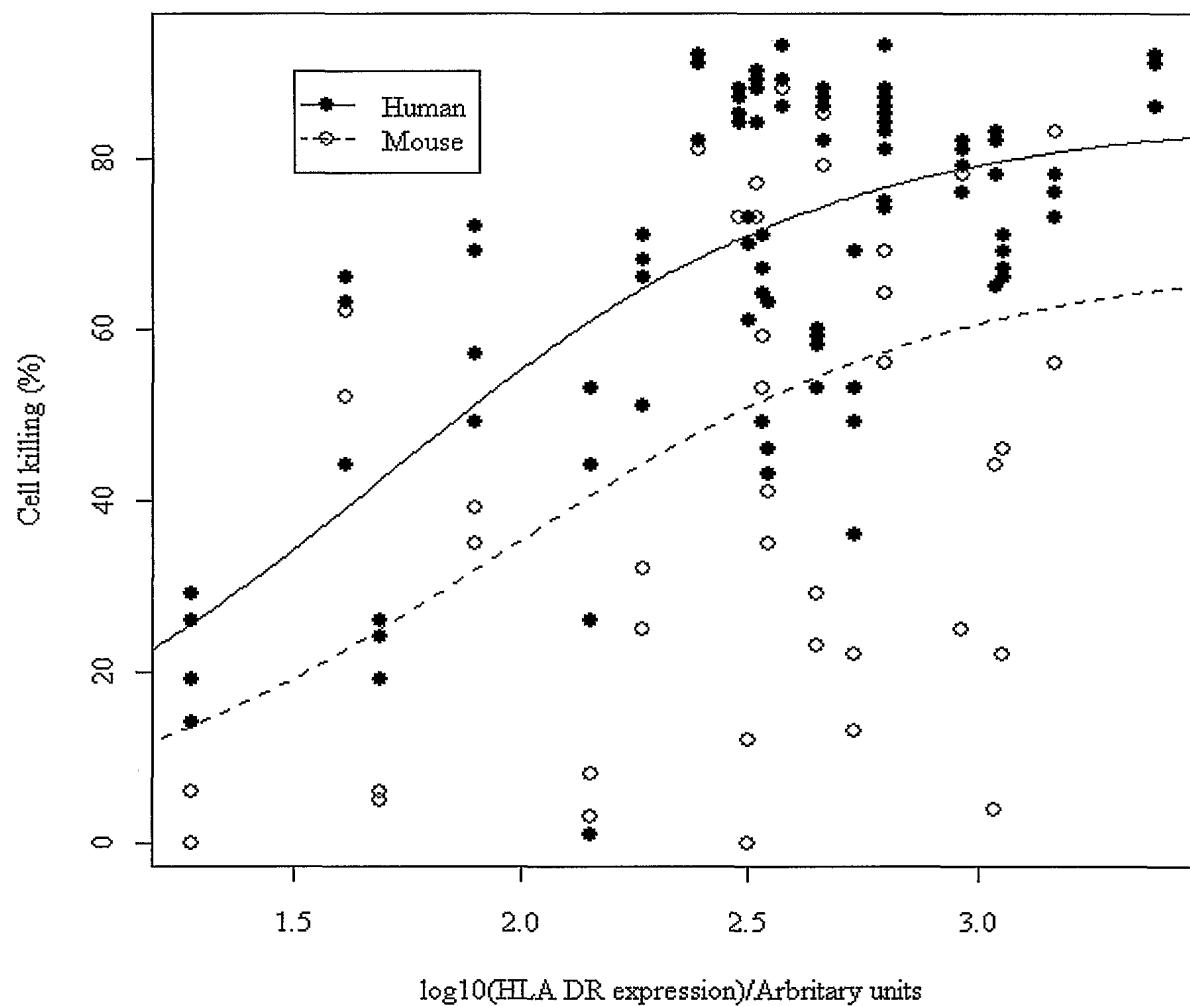
Figure 4

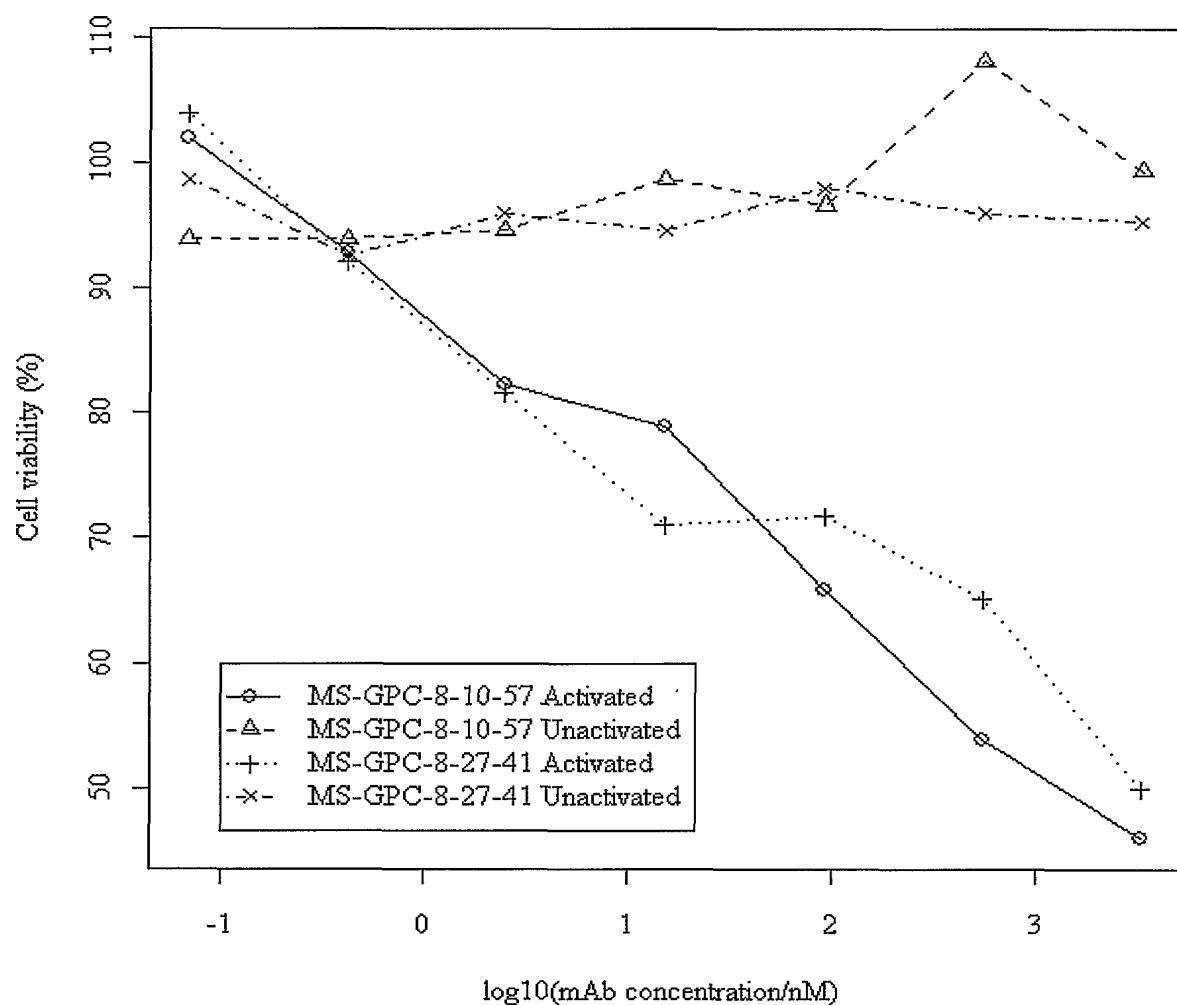
Figure 5

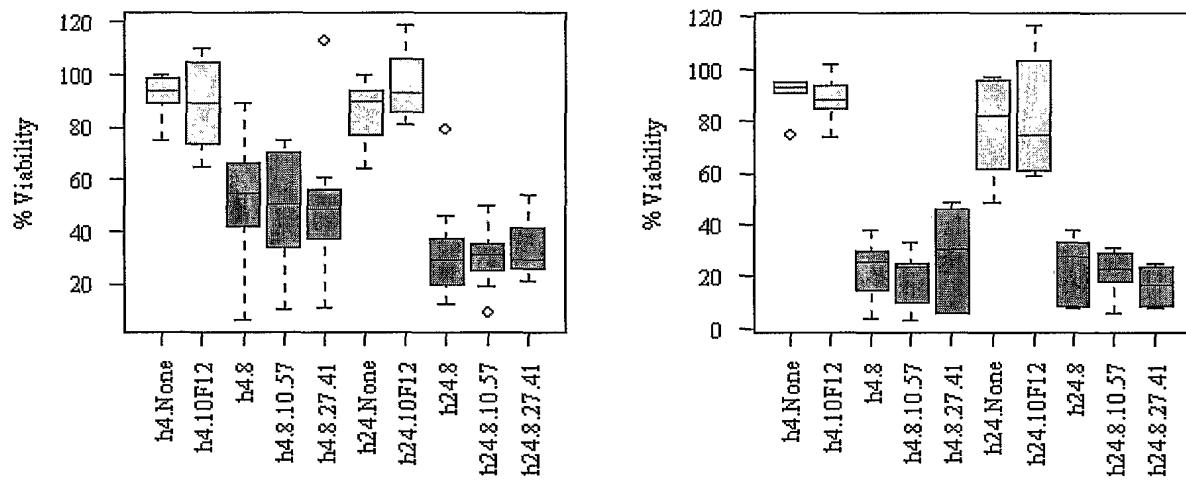
Figure 6

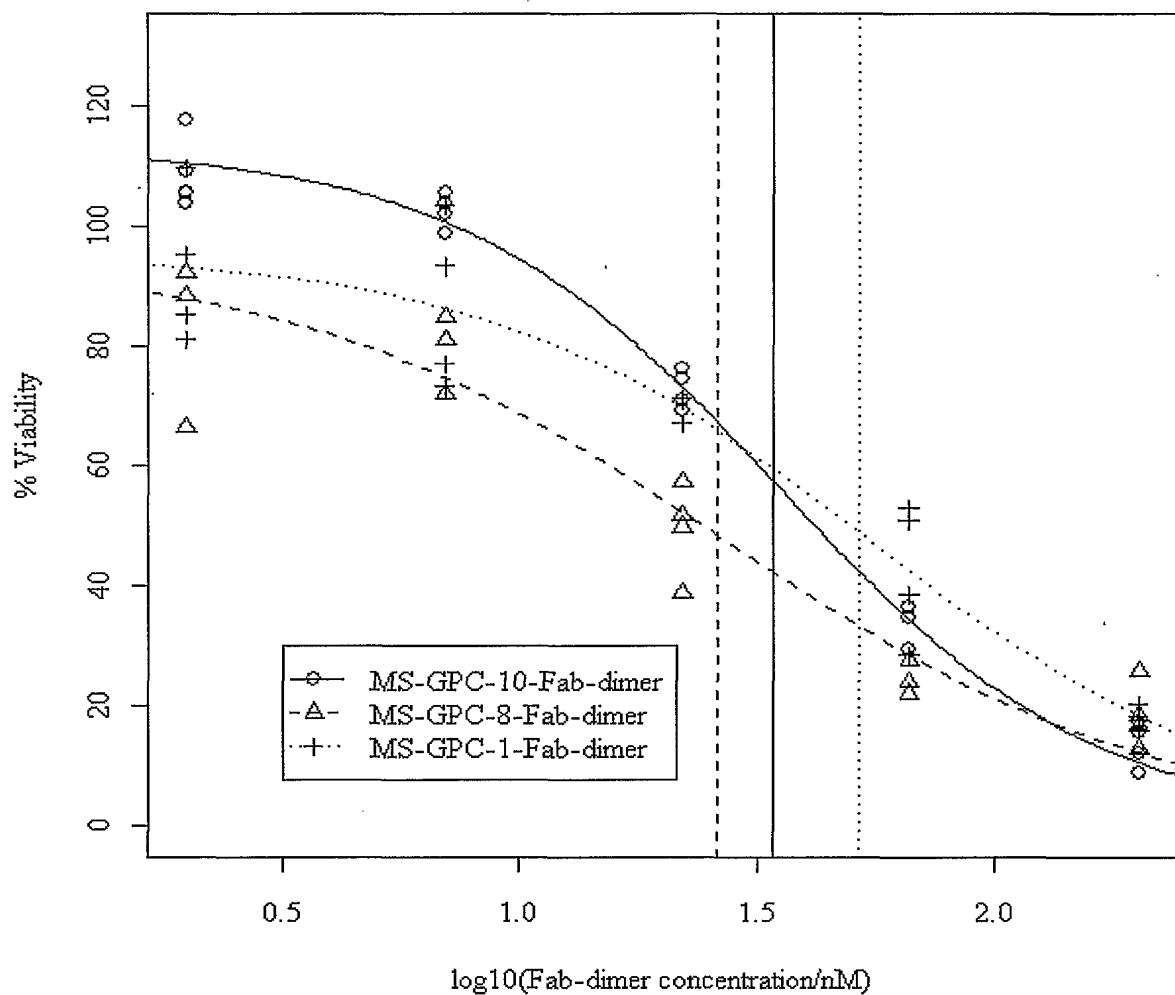
Figure 7a

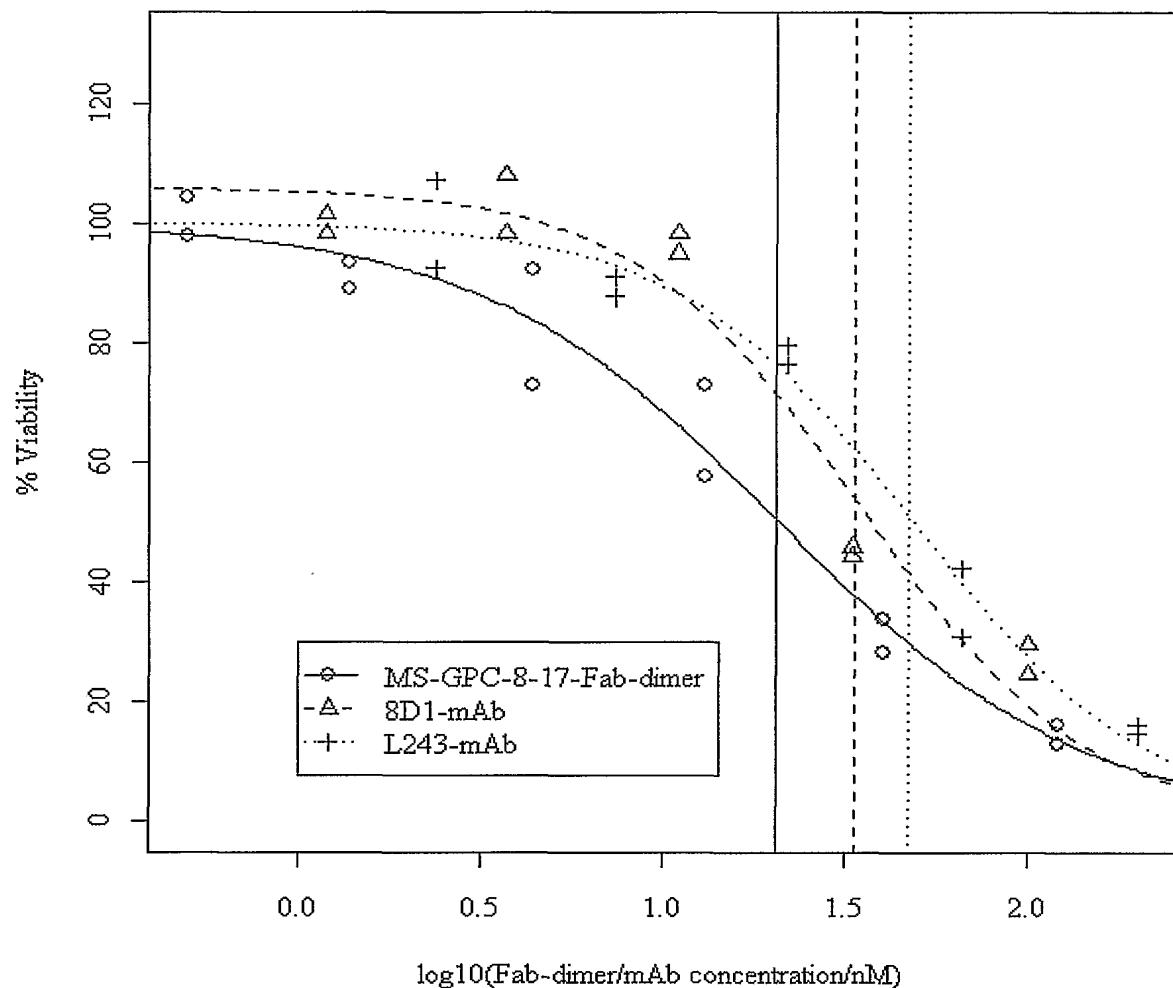
Figure 7b

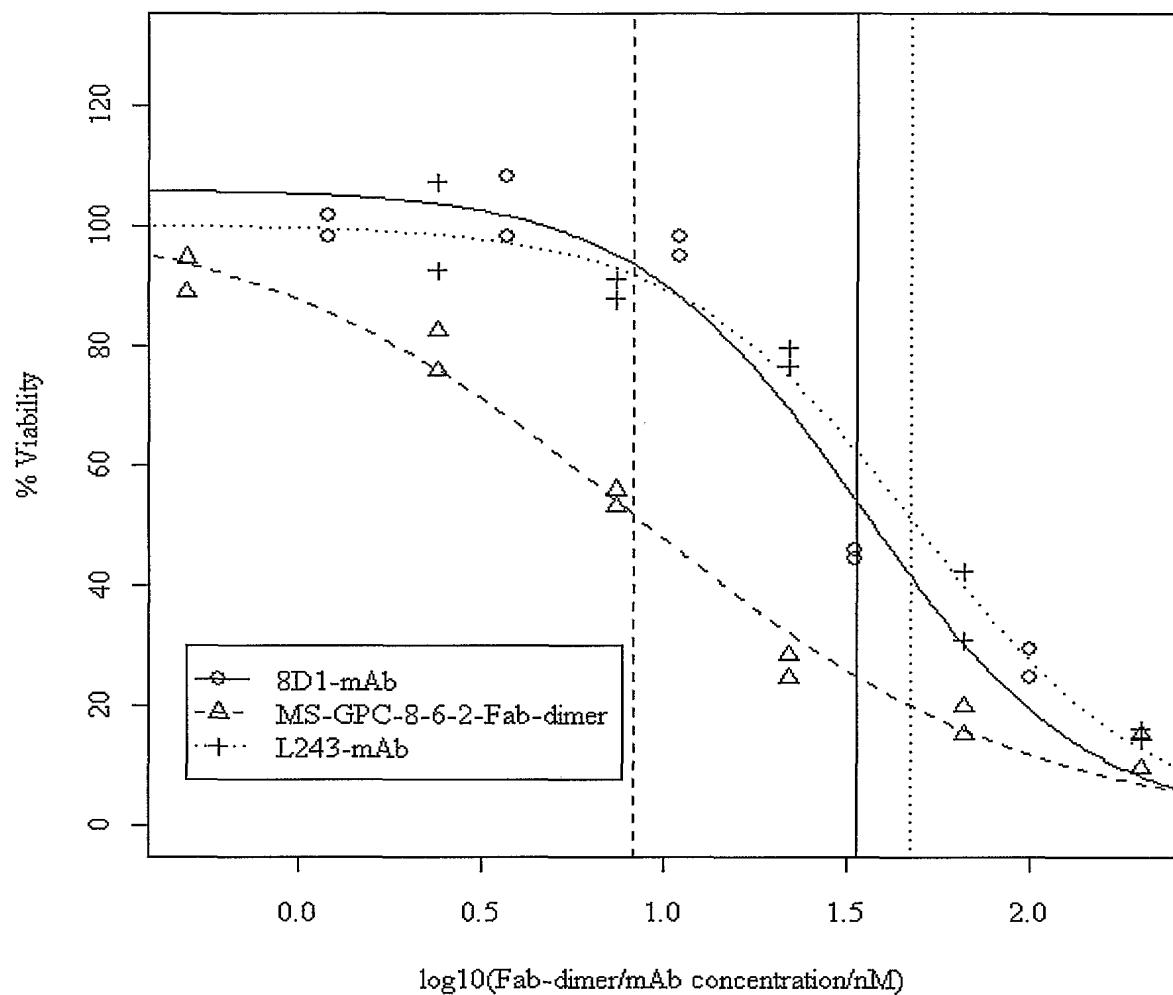
Figure 7c

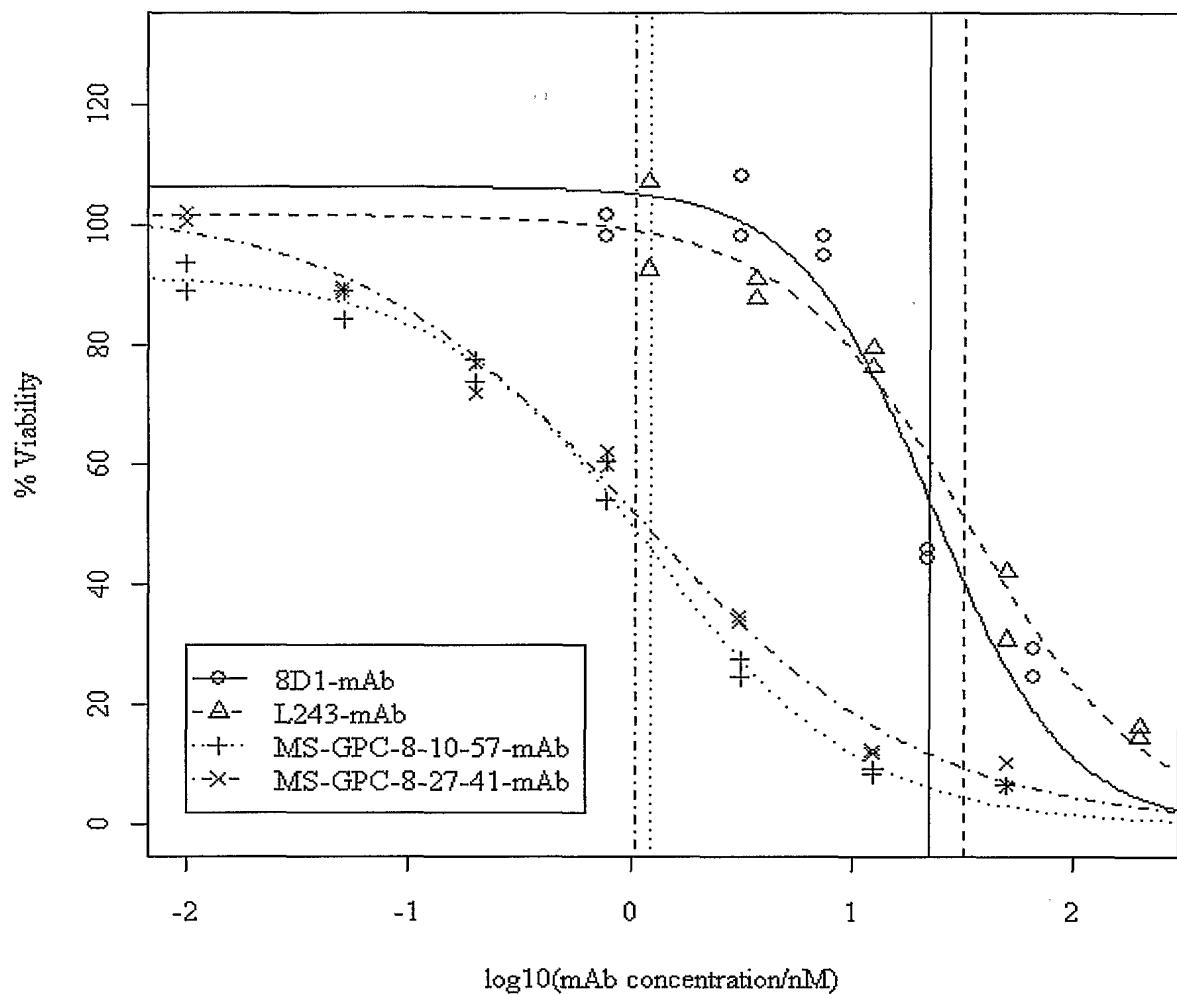
Figure 7d

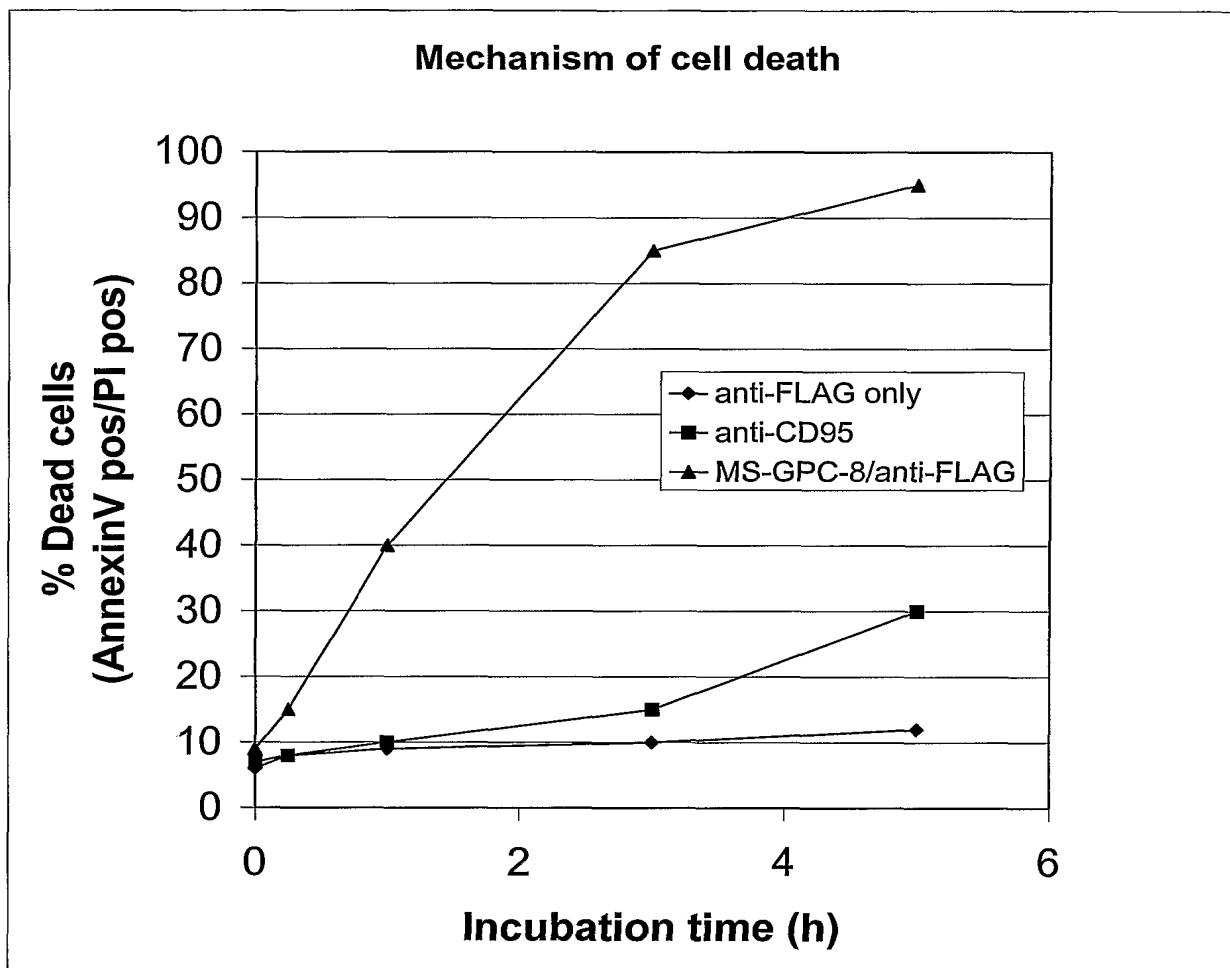
Figure 8a

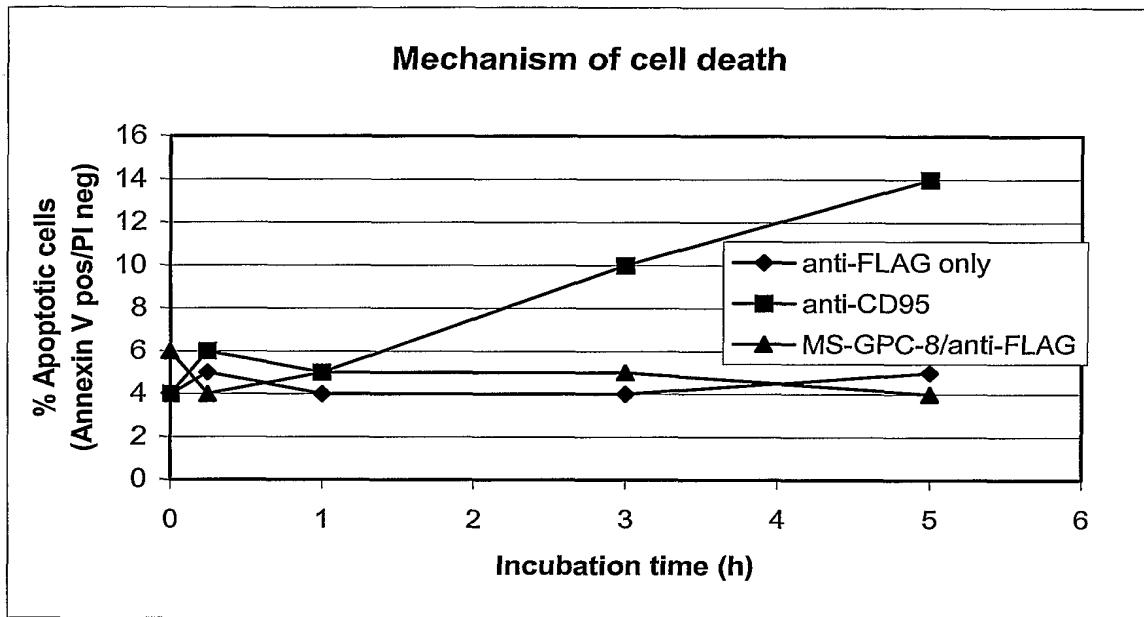
Figure 8b

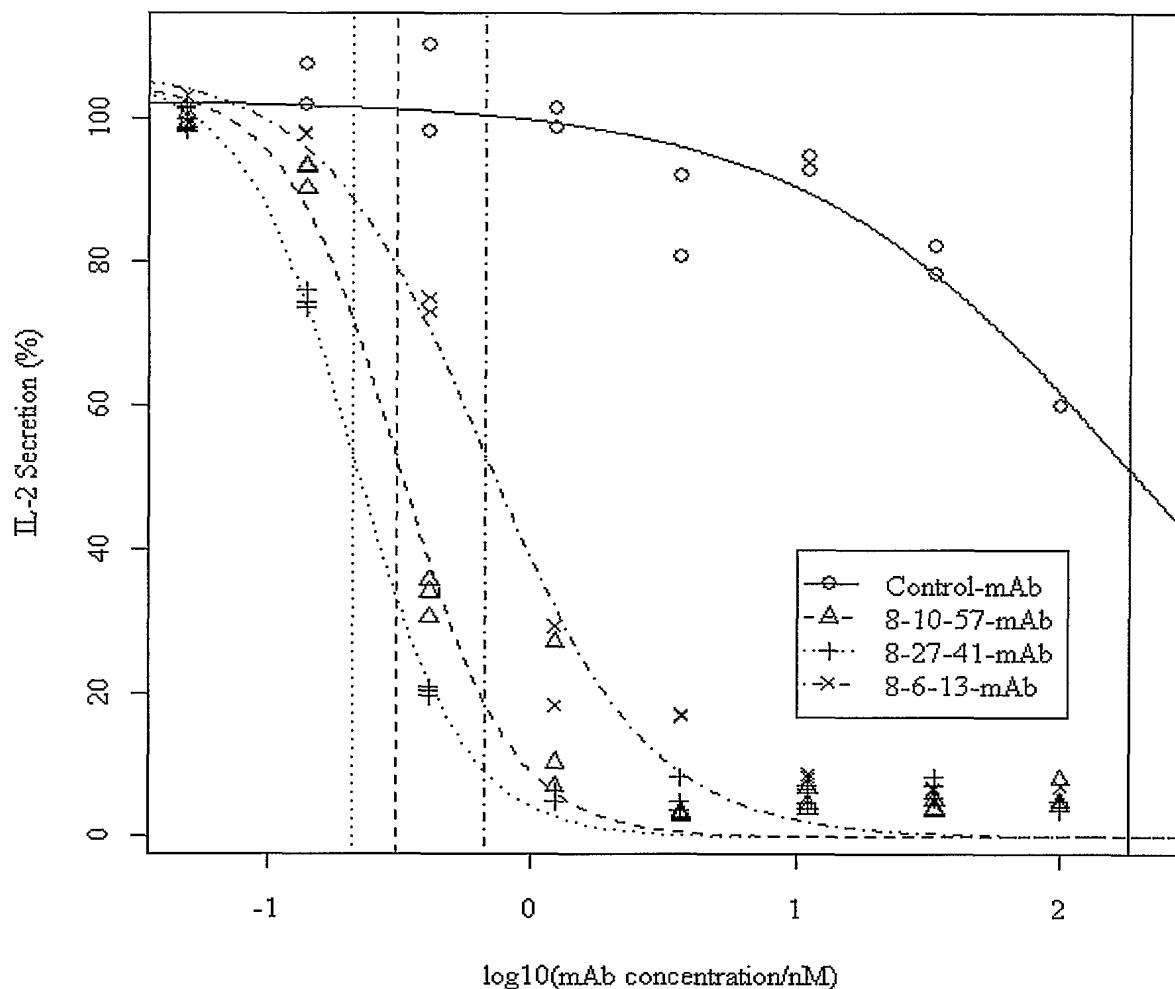
Figure 9a

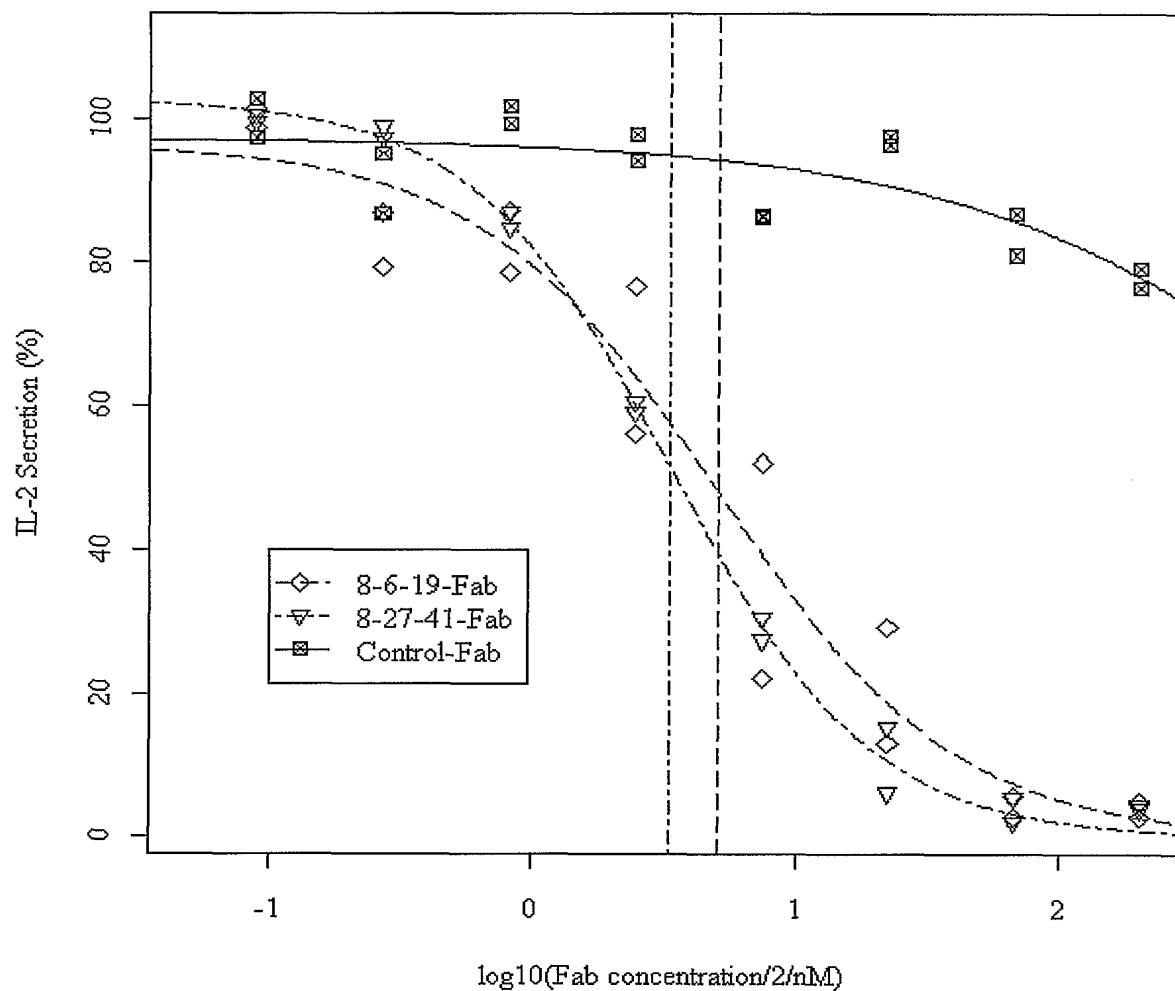
Figure 9b

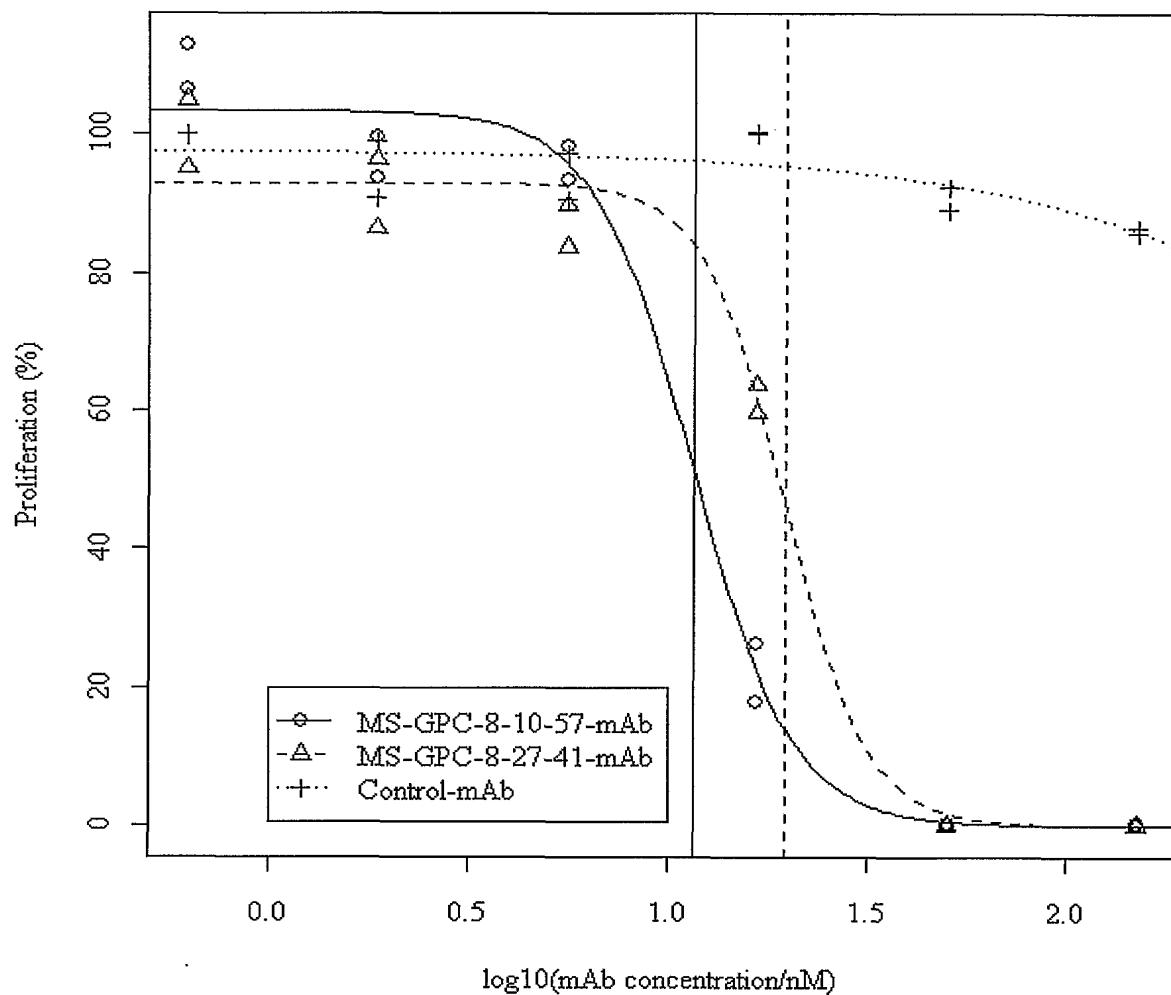
Figure 10

Figure 11

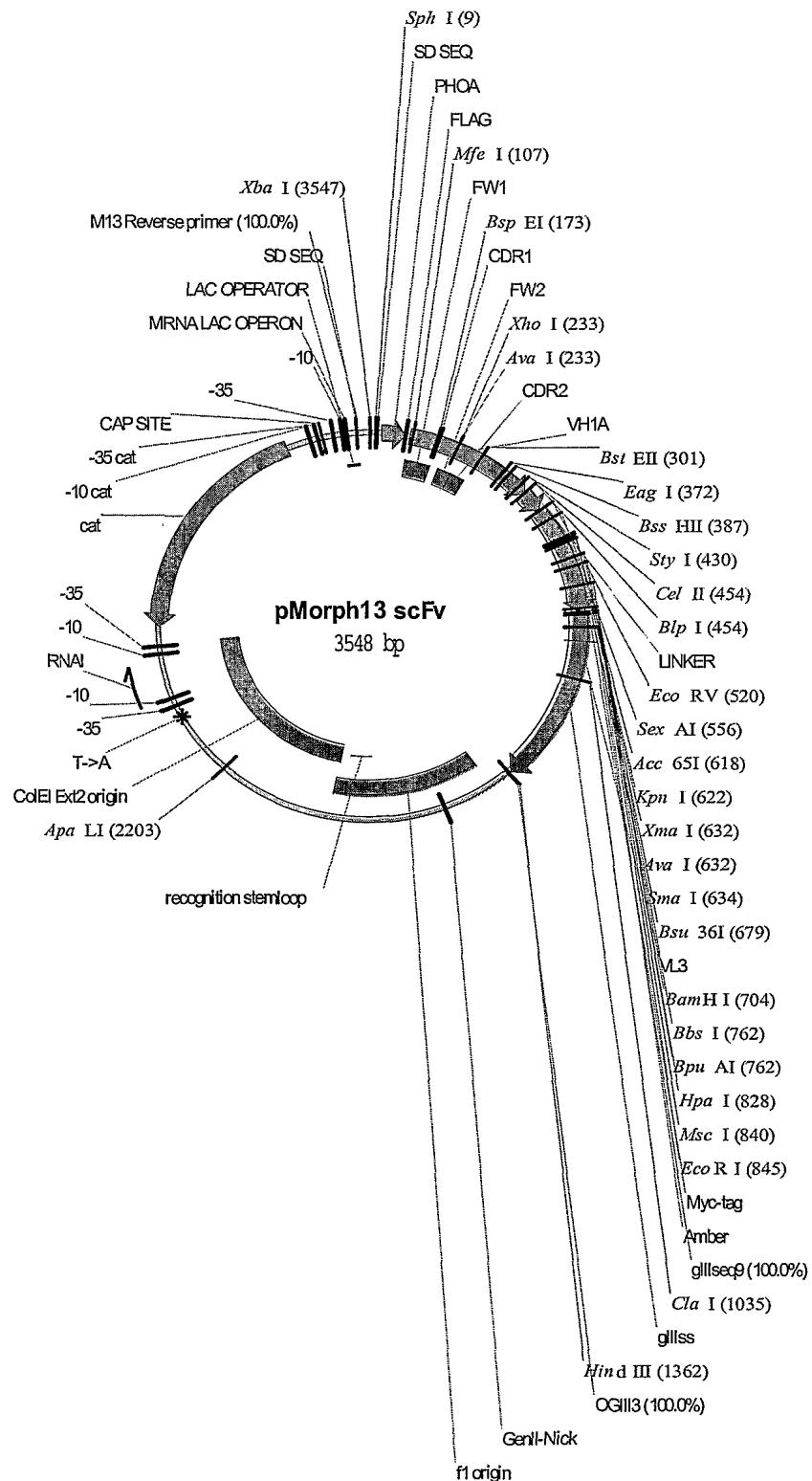


Figure 11 (cont)

XbaI SphI

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1 AGAGCATGCG TAGGAGAAAA TAAAATGAAA CAAAGCACTA TTGCACTGGC  
TCTCGTACGC ATCCTCTTT ATTTTACTTT GTTTCGTGAT AACGTGACCG

51 ACTCTTACCG TTGCTCTTCA CCCCTGTTAC CAAAGCCGAC TACAAAGATG  
TGAGAATGGC AACGAGAAGT GGGGACAATG GTTTCGGCTG ATGTTTCTAC

MfeI

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101 AAGTGCAATT GGTTCAAGTCT GGCGCGGAAG TGAAAAAAACC GGGCAGCAGC
TTCACGTTAA CCAAGTCAGA CCGCGCCTTC ACTTTTTGG CCCGTCGTCG

BspEI

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151 GTGAAAGTGA GCTGCAAAGC CTCCGGAGGC ACTTTTAGCA GCTATGCGAT  
CACTTTCACT CGACGTTTCG GAGGCCTCCG TGAAAATCGT CGATACGCTA

XbaI

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AvaI

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201 TAGCTGGGTG CGCCAAGCCC CTGGGCAGGG TCTCGAGTGG ATGGGCGGCA  
ATCGACCCAC GCGGTTCGGG GACCCGTCCC AGAGCTCACC TACCCGCCGT

BstEII

~

251 TTATTCCGAT TTTTGGCACG GCGAACTACG CGCAGAAGTT TCAGGGCCGG  
AATAAGGCTA AAAACCGTGC CGCTTGATGC GCGTCTCAA AGTCCC GCC

BstEII

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301 GTGACCATT A CCGCGGATGA AAGCACCAGC ACCCGCGTATA TGGAACTGAG
CACTGGTAAT GGCGCCTACT TTCGTGGTCG TGGCGCATAT ACCTTGACTC

EagI

BssHII

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351 CAGCCTGCGT AGCGAAGATA CGGCCGTGTA TTATTGCGCG CGTTATTATG
GTCGGACGCA TCGCTTCTAT GCCGGCACAT AATAACGCGC GCAATAATAC

StyI

~~~~~

401 ATCGTATGTA TAATATGGAT TATTGGGCC AAGGCACCCCT GGTGACGGTT  
TAGCATAACAT ATTATACCTA ATAACCCGG TTCCGTGGGA CCACTGCCAA

BlnI

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CelII

~~~~~

451 AGCTCAGCGG GTGGCGGTTC TGGCGCGGT GGGAGCGGTG GCGGTGGTTC  
TCGAGTCGCC CACCGCCAAG ACCGCCGCCA CCCTCGCCAC CGCCACCAAG

EcoRV

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501 TGGCGGTGGT GGTTCCGATA TCGAACTGAC CCAGCCGCCT TCAGTGAGCG

ACCGCCACCA CCAAGGCTAT AGCTTGACTG GGTCGGCGGA AGTCACTCGC

SexAI

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551 TTGCACCAGG TCAGACCGCG CGTATCTCGT GTAGCGGCGA TGCGCTGGC  
AACGTGGTCC AGTCTGGCGC GCATAGAGCA CATCGCCGCT ACGCGACCCG

XmaI

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KpnI

SmaI

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Acc65I

AvaI

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601 GATAAAATACG CGAGCTGGTA CCAGCAGAAA CCCGGGCAGG CGCCAGTTCT
CTATTTATGC GCTCGACCAT GGTCGTCTTT GGGCCCGTCC GCGGTCAAGA

Bsu36I

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651 GGTGATTAT GATGATTCTG ACCGTCCCTC AGGCATCCCG GAACGCTTTA  
CCACTAAATA CTACTAAGAC TGGCAGGGAG TCCGTAGGGC CTTGCGAAAT

BamHI

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701 GCGGATCCAA CAGCGGCAAC ACCCGCACCC TGACCATTAG CGGCACTCAG
CGCCTAGGTT GTCGCCGTTG TGGCGCTGGG ACTGGTAATC GCCGTGAGTC

BpuAI

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BbsI

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751 GCGGAAGACG AAGCGGATTA TTATTGCCAG AGCTATGACG CTCATATGCG
CGCCTCTGC TTCCCTTAAT AATAACGGTC TCGATACTGC GAGTATACGC

HpaI

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MscI

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ECORI

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801 TCCTGTGTTT GGCGCGGGCA CGAAGTTAAC CGTTCTGGC CAGGAATTG  
AGGACACAAA CCGCCGCCGT GCTTCAATTG GCAAGAACCG GTCCTTAAGC

851 AGCAGAAGCT GATCTCTGAG GAGGATCTGA ACTAGGGTGG TGGCTCTGGT  
TCGTCTTCGA CTAGAGACTC CTCCTAGACT TGATCCCACC ACCGAGACCA

901 TCCGGTGATT TTGATTATGA AAAGATGGCA AACGCTAATA AGGGGGCTAT  
AGGCCACTAA AACTAATACT TTTCTACCGT TTGCGATTAT TCCCCCGATA  
gIIIseq9 100.0%

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951 GACCGAAAAT GCCGATGAAA ACGCGCTACA GTCTGACGCT AAAGGCAAAC  
CTGGCTTTA CGGCTACTTT TGCGCGATGT CAGACTGCGA TTTCCGTTG

ClaI

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1001 TTGATTCTGT CGCTACTGAT TACGGTGCTG CTATCGATGG TTTCATGGT
AACTAAGACA GCGATGACTA ATGCCACGAC GATAGCTACC AAAGTAACCA

1051 GACGTTTCCG GCCTTGCTAA TGGTAATGGT GCTACTGGTG ATTTTGCTGG
CTGCAAAGGC CGGAACGATT ACCATTACCA CGATGACCCAC TAAAACGACC

20/49

1101 CTCTAATTCC CAAATGGCTC AAGTCGGTGA CGGTGATAAT TCACCTTTAA
GAGATTAAGG GTTTACCGAG TTCAGCCACT GCCACTATTA AGTGGAAATT

1151 TGAATAATT TTACCTTCCC TCCCTCAATC GGTTGAATGT
ACTTATTAAA GGCAGTTATA AATGGAAGGG AGGGAGTTAG CCAACTTACA

1201 CGCCCTTTG TCTTGCGC TGGTAAACCA TATGAATT TTATTGATTG
GCGGGAAAAC AGAAACCGCG ACCATTTGGT ATACTTAAAA GATAACTAAC

1251 TGACAAAATA AACTTATTCC GTGGTGTCTT TGCCTTCTT TTATATGTTG
ACTGTTTAT TTGAATAAGG CACCACAGAA ACGCAAAGAA AATATACAAAC

1301 CCACCTTTAT GTATGTATTT TCTACGTTTG CTAACATACT GCGTAATAAG
GGTGGAAATA CATACTAAA AGATGCAAAC GATTGTATGA CGCATTATTC

HindIII

~~~~~

1351 GAGTCTTGAT AAGCTTGACC TGTGAAGTGA AAAATGGCGC AGATTGTGCG  
CTCAGAACTA TTCGAACCTGG ACACCTCACT TTTTACCGCG TCTAACACGC  
OGIII3 100.0%

=====

1401 ACATTTTTT TGTCTGCCGT TTAATGAAAT TGTAAACGTT AATATTTGT  
TGAAAAAAA ACAGACGGCA AATTACTTTA ACATTTGCAA TTATAAAACA

1451 TAAAATTCGC GTTAAATTTT TGTTAAATCA GTCATTTTT TAACCAATAG  
ATTTAAGCG CAATTAAAA ACAATTAGT CGAGTAAAAA ATTGGTTATC

1501 GCCGAAATCG GCAAAATCCC TTATAATCA AAAGAATAGA CCGAGATAGG  
CGGCTTAGC CGTTTAGGG AATATTAGT TTTCTTATCT GGCTCTATCC

1551 GTTGAGTGT GTTCCAGTTT GGAACAAGAG TCCACTATTA AAGAACGTGG  
CAACTCACAA CAAGTCAAA CCTTGTCTC AGGTGATAAT TTCTTGCACC

1601 ACTCCAACGT CAAAGGGCGA AAAACCGTCT ATCAGGGCGA TGGCCCACTA  
TGAGGTTGCA GTTTCCCGCT TTTTGGCAGA TAGTCCCGCT ACCGGGTGAT

1651 CGAGAACCAT CACCTTAATC AAGTTTTTG GGGTCGAGGT GCCGTAAGC  
GCTCTGGTA GTGGGATTAG TTCAAAAAAC CCCAGCTCCA CGGCATTCG

1701 ACTAAATCGG AACCTAAAG GGAGCCCCG ATTTAGAGCT TGACGGGAA  
TGATTTAGCC TTGGGATTTC CCTCGGGGGC TAAATCTCGA ACTGCCCTT

1751 AGCCGGCGAA CGTGGCGAGA AAGGAAGGGA AGAAAGCGAA AGGAGCGGGC  
TCGGCCGCTT GCACCGCTCT TTCCCTCCCT TCTTCGCTT TCCTCGCCG

1801 GCTAGGGCGC TGGCAAGTGT AGCGGTACG CTGCGCGTAA CCACCACACC  
CGATCCCGCG ACCGTTACA TCGCCAGTGC GACGCGCATT GGTGGTGTGG

1851 CGCCGCGCTT AATGCGCCGC TACAGGGCGC GTGCTAGCCA TGTGAGCAA  
CGGGCGCGAA TTACCGGGCG ATGTCCCGCG CACGATCGGT ACACTCGTT

1901 AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA GGCGCGTTG CTGGCGTTT  
TCCGGTCGTT TTCCGGTCCT TGGCATT TTTT CCGGCGAAC GACCGAAAAA

1951 TCCATAGGCT CCGCCCCCCT GACGAGCATC AAAAAATCG ACGCTCAAGT  
AGGTATCCGA GGCGGGGGG A CTGCTCGTAG TGTTTTAGC TGCGAGTTCA

2001 CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC  
GTCTCCACCG CTGGGGCTG TCCTGATATT TCTATGGTCC GCAAAGGGGG  
2051 TGGAAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT  
ACCTTCGAGG GAGCACGCGA GAGGACAAGG CTGGGACGGC GAATGGCCTA  
2101 ACCTGTCCGC CTTTCTCCCT TCGGGAAAGCG TGGCGCTTC TCATAGCTCA  
TGGACAGGGC GAAAGAGGGA AGCCCTTCGC ACCCGAAAG AGTATCGAGT  
2151 CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG  
GCGACATCCA TAGAGTCAAG CCACATCCAG CAAGCGAGGT TCGACCCGAC

## ApalI

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2201 TGTGCACGAA CCCCCCGTTC AGTCCGACCG CTGCGCCTTA TCCGGTAAC
ACACGTGCTT GGGGGGCAAG TCAGGCTGGC GACGCGGAAT AGGCCATTGA
2251 ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA
TAGCAGAACT CAGGGTGGGC CATTCTGTGC TGAATAGCGG TGACCGTCGT
2301 GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA
CGGTGACCAT TGTCTTAATC GTCTCGCTCC ATACATCCGC CACGATGTCT
2351 GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGA ACAGTATTG
CAAGAACTTC ACCACCGGAT TGATGCCGAT GTGATCTTCT TGTCAAAAC
2401 GTATCTGCGC TCTGCTGTAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC
CATAGACGCG AGACGACATC GGTCAATGGA AGCCTTTTC TCAACCATCG
2451 TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTGTTTG
AGAACTAGGC CGTTGTTTG GTGGCGACCA TCGCCACCAA AAAAACAAAC
2501 CAAGCAGCAG ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTGA
GTTCGTCGTC TAATGCGCGT CTTTTTTCC TAGAGTTCTT CTAGGAAACT
2551 TCTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC ACGTTAACGG
AGAAAAGATG CCCCAGACTG CGAGTCACCT TGCTTTGAG TGCAATTCCC
2601 ATTTTGGTCA GATCTAGCAC CAGGCCTTTA AGGGCACCAA TAACTGCCTT
TAAAACCAGT CTAGATCGTG GTCCGCAAAT TCCCGTGGTT ATTGACGGAA
2651 AAAAAAATTAA CGCCCCGCC TGCCACTCAT CGCAGTACTG TTGTAATTCA
TTTTTTAAT GCGGGGCGGG ACGGTGAGTA GCGTCATGAC AACATTAAGT
2701 TTAAGCATTG TGCCGACATG GAAGCCATCA CAAACGGCAT GATGAACCTG
AATTGTAAG ACGGCTGTAC CTTCGGTAGT GTTGCCGTA CTACTTGGAC
2751 AATCGCCAGC GGCATCAGCA CCTTGTGCC TTGCGTATAA TATTGCCA
TTAGCGGTGCG CCGTAGTCGT GGAACAGCGG AACGCATATT ATAAACGGGT
2801 TAGTGAAAAC GGGGGCGAAG AAGTTGTCCA TATTGGCTAC GTTTAAATCA
ATCACTTTG CCCCCGCTTC TTCAACAGGT ATAACCGATG CAAATTTAGT
2851 AAACTGGTGA AACTCACCCA GGGATTGGCT GAGACGAAAA ACATATTCTC
TTGACCACT TTGAGTGGGT CCCTAACCGA CTCTGCTTT TGTATAAGAG

22/49

2901 AATAAACCCCT TTAGGGAAAT AGGCCAGGTT TTCACCGTAA CACGCCACAT
 TTATTGGGA AATCCCTTA TCCGGTCAA AAGTGGCATT GTGCGGTGTA

 2951 CTTGCGAATA TATGTGTAGA AACTGCCGA AATCGTCGTG GTATTCACTC
 GAACGCTTAT ATACACATCT TTGACGGCCT TTAGCAGCAC CATAAGTGAG

 3001 CAGAGCGATG AAAACGTTTC AGTTGCTCA TGGAAAACGG TGTAACAAGG
 GTCTCGCTAC TTTGCAAAG TCAAACGAGT ACCTTTGCC ACATTGTTCC

 3051 GTGAACACTA TCCCATATCA CCAGCTCACC GTCTTCATT GCCATACGGA
 CACTTGTGAT AGGGTATAGT GGTCGAGTGG CAGAAAGTAA CGGTATGCCT

 3101 ACTCCGGGTG AGCATTCATC AGGCGGCAA GAATGTGAAT AAAGGCCGGA
 TGAGGCCAC TCGTAAGTAG TCCGCCCGTT CTTACACTTA TTTCCGGCCT

 3151 TAAAACTTGT GCTTATTTTT CTTTACGGTC TTTAAAAAGG CCGTAATATC
 ATTTGAACA CGAATAAAAAA GAAATGCCAG AAATTTTCC GGCATTATAG

 3201 CAGCTGAACG GTCTGGTTAT AGGTACATTG AGCAACTGAC TGAAATGCCT
 GTCGACTTGC CAGACCAATA TCCATGTAAC TCGTTGACTG ACTTTACGGA

 3251 CAAAATGTTT CTTACGATGC CATTGGATA TATCAACGGT GGTATATCCA
 GTTTACAAG AAATGCTACG GTAACCCTAT ATAGTTGCCA CCATATAGGT

 3301 GTGATTTTTT TCTCCATTAA AGCTTCCTTA GCTCCTGAAA ATCTGATAA
 CACTAAAAAA AGAGGTAAAA TCGAAGGAAT CGAGGACTTT TAGAGCTATT

 3351 CTCAAAAAAT ACGCCCGGTA GTGATCTTAT TTCATTATGG TGAAAGTTGG
 GAGTTTTTA TGCGGGCCAT CACTAGAATA AAGTAATACC ACTTTCAACC

 3401 AACCTCACCC GACGTCTAAT GTGAGTTAGC TCACTCATTA GGCACCCAG
 TTGGAGTGGG CTGCAGATTA CACTCAATCG AGTGAGTAAT CCGTGGGTC

 3451 GCTTACACT TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG
 CGAAATGTGA AATACGAAGG CCGAGCATAAC AACACACCTT AACACTCGCC

 M13 Reverse primer 100.0% XbaI
 ===== ~
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 TATTGTTAAA GTGTGTCCTT TGTCGATACT GGTACTAATG CTTAAAGA

Figure 12

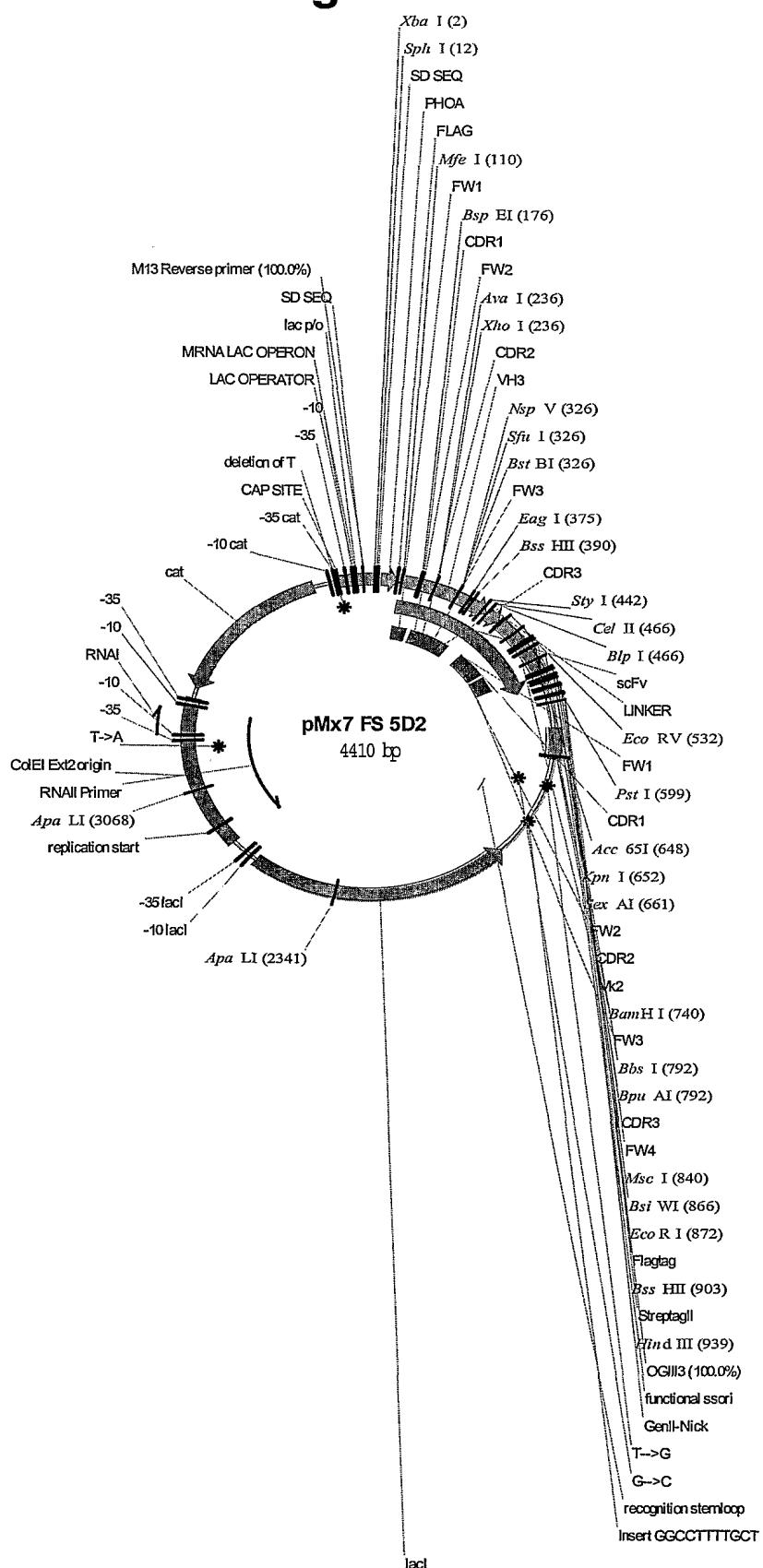


Figure 12 (cont)

XbaI SphI
~~~~~

1 TCTAGAGCAT GCGTAGGAGA AAATAAAATG AAACAAAGCA CTATTGCACT  
AGATCTCGTA CGCATCCTCT TTTATTTAC TTTGTTTCGT GATAACGTGA

51 GGCACTCTTA CCGTGCTCT TCACCCCTGT TACCAAAGCC GACTACAAAG  
CCGTGAGAAT GGCAACGAGA AGTGGGGACA ATGGTTTCGG CTGATGTTTC

MfeI  
~~~~~

101 ATGAAGTGCA ATTGGTGGAA AGCGGCGGCG GCCTGGTGCA ACCGGGCGGC
TACTTCACGT TAACCACCTT TCGCCGCCGC CGGACCACGT TGGCCCGCCG

BspEI
~~~~~

151 AGCCTGCGTC TGAGCTGCGC GGCCTCCGGA TTTACCTTTA GCAGCTATGC  
TCGGACGCAG ACTCGACGCG CCGGAGGCCT AAATGGAAAT CGTCGATACG

XhoI  
~~~~~

201 GATGAGCTGG GTGCGCCAAG CCCCTGGAA GGGTCTCGAG TGGGTGAGCG
CTACTCGACC CACCGGGTTC GGGGACCCCTT CCCAGAGCTC ACCCACTCGC

251 CGATTAGCGG TAGCGGCGGC AGCACCTATT ATGCGGATAG CGTGAAAGGC
GCTAATCGCC ATCGCCGCCG TCGTGGATAA TACGCCTATC GCACTTTCCG

AvaI
~~~~~

201 GATGAGCTGG GTGCGCCAAG CCCCTGGAA GGGTCTCGAG TGGGTGAGCG  
CTACTCGACC CACCGGGTTC GGGGACCCCTT CCCAGAGCTC ACCCACTCGC

251 CGATTAGCGG TAGCGGCGGC AGCACCTATT ATGCGGATAG CGTGAAAGGC  
GCTAATCGCC ATCGCCGCCG TCGTGGATAA TACGCCTATC GCACTTTCCG

BstBI  
~~~~~

301 CGTTTACCA TTTCACGTGA TAATTGAAA AACACCCGT ATCTGCAAAT
GCAAAATGGT AAAGTGCACT ATTAAGCTTT TTGTGGGACA TAGACGTTTA

SfuI
~~~~~

351 GAACAGCCTG CGTGGAAAG ATACGGCCGT GTATTATTGC GCGCGTGTAA  
CTTGTGGAC GCACGCCCTTC TATGCCGGCA CATAATAACG CGCGCACAAAT

NspV  
~~~~~

301 CGTTTACCA TTTCACGTGA TAATTGAAA AACACCCGT ATCTGCAAAT
GCAAAATGGT AAAGTGCACT ATTAAGCTTT TTGTGGGACA TAGACGTTTA

EagI BssHII
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351 GAACAGCCTG CGTGGAAAG ATACGGCCGT GTATTATTGC GCGCGTGTAA  
CTTGTGGAC GCACGCCCTTC TATGCCGGCA CATAATAACG CGCGCACAAAT

StyI  
~~~~~

401 AGAACGATTG TTCTCGTAAG AATTGGTTTG ATTATTGGGG CCAAGGCACC
TCTTCGTAAA AAGAGCATTG TTAACCAAAC TAATAACCCC GGTTCCGTGG

BpuI
~~~~~  
CelII  
~~~~~

451 CTGGTGACGG TTAGCTCAGC GGGTGGCGGT TCTGGCGGCG GTGGGAGCGG
GACCACTGCC AATCGAGTCG CCCACCGCCA AGACCGCCGC CACCCTCGCC

EcoRV
~~~~~

501 TGGCGGTGGT TCTGGCGGTG GTGGTTCCGA TATCGTGATG ACCCAGAGCC  
ACCGCCACCA AGACCGCCAC CACCAAGGCT ATAGCACTAC TGGGTCTCGG

PstI  
~~~~~  
Acc65I
~~~~~

551 CACTGAGCCT GCCAGTGACT CGGGCGAGC CTGCGAGCAT TAGCTGCAGA  
GTGACTCGGA CGGTCACTGA GGCCCCTCG GACGCTCGTA ATCGACGTCT

KpnI  
~~~  
Acc65I
~~~

601 AGCAGCCAAA GCCTGCTGCA TAGCAACGGC TATAACTATC TGGATTGGTA  
TCGTCGGTTT CGGACGACGT ATCGTTGCCG ATATTGATAG ACCTAACCAT

KpnI  
~~~  
Acc65I SexAI
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651 CCTTCAAAAA CCAGGTCAAA GCCCGCAGCT ATTAATTAT CTGGGCAGCA  
GGAAGTTTTT GGTCCAGTTT CGGGCGTCGA TAATTAAATA GACCCGTCGT

BamHI  
~~~~~

701 ACCGTGCCAG TGGGGTCCCG GATCGTTTA GCGGCTCTGG ATCCGGCACC
TGGCACGGTC ACCCCAGGGC CTAGCAAAT CGCCGAGACC TAGGCCGTGG

BpuAI
~~~~~  
BbsI  
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751 GATTTACCC TGAAAATTAG CCGTGTGGAA GCTGAAGACG TGGGCGTGT
CTAAAATGGG ACTTTAACAT GGCACACCTT CGACTTCTGC ACCCGCACAT

Mscl
~~~~~

801 TTATTGCCAG CAGCATTATA CCACCCCGCC GACCTTGGC CAGGGTACGA  
AATAACGGTC GTCGTAATAT GGTGGGGCGG CTGGAAACCG GTCCCATGCT

BsiWI EcoRI

|      |                                                                                                                                |                |
|------|--------------------------------------------------------------------------------------------------------------------------------|----------------|
| 851  | AAGTTGAAAT TAAACGTACG GAATTGACT ATAAAGATGA CGATGACAAA<br>TTCAACTTTA ATTTGCATGC CTTAAGCTGA TATTCTACT GCTACTGTTT                 |                |
|      |                                                                                                                                | BssHII HindIII |
| 901  | GGCGCGCCGT GGAGGCCACCC GCAGTTTGAA AAATGATAAG CTTGACCTGT<br>CCGCGCGGCA CCTCGGTGGG CGTCAAAC TTACTATTC GAACTGGACA<br>OGIII 100.0% |                |
|      |                                                                                                                                | =====          |
| 951  | GAAGTGAAAA ATGGCGCAGA TTGTGCGACA TTTTTTTGT CTGCCGTTA<br>CTTCACCTTT TACCGCGTCT AACACGCTGT AAAAAAAACA GACGGCAAAT<br>OGIII 100.0% |                |
|      |                                                                                                                                | =====          |
| 1001 | ATTAAGGGGG GGGGGGGGCC GCCCTGGGGG GGGGTGTACA TGAAATTGTA<br>TAATTCCCCC CCCCCCCCCGG CC GGACCCCC CCCCACATGT ACTTTAACAT             |                |
| 1051 | AACGTTAATA TTTGTTAAA ATTGCGTTA AATTTTGTT AAATCAGCTC<br>TTGCAATTAT AAAACAATT TAAGCGCAAT TTAAAAACAA TTTAGTCGAG                   |                |
| 1101 | ATTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT AAATCAAAAG<br>TAAAAAATTG GTTATCCGGC TTTAGCCGTT TTAGGGATA TTTAGTTTC                  |                |
| 1151 | AATAGACCGA GATAGGGTTG AGTGTGTTTC CAGTTGGAA CAAGAGTCCA<br>TTATCTGGCT CTATCCCAAC TCACAACAAG GTCAAACCTT GTTCTCAGGT                |                |
| 1201 | CTATTAAAGA ACGTGGACTC CAACGTCAA GGGCGAAAAA CCGTCTATCA<br>GATAATTCT TGCACCTGAG GTTGCAGTTT CCCGCTTTT GGCAGATAGT                  |                |
| 1251 | GGCGATGGC CCACTACGAG AACCATCACC CTAATCAAGT TTTTGGGGT<br>CCCGCTACCG GGTGATGCTC TTGGTAGTGG GATTAGTTCA AAAAACCCCA                 |                |
| 1301 | CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG CCCCCGATTT<br>GCTCCACGGC ATTCGTTGAT TTAGCCTTGG GATTTCCCTC GGGGGCTAAA               |                |
| 1351 | AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA<br>TCTCGAACTG CCCCTTTCGG CCGCTTGCAC CGCTCTTCC TTCCCTTCTT                |                |
| 1401 | AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG GTCACGCTGC<br>TCGCTTCCCT CGCCCGCGAT CCCGCGACCG TTCACATCGC CAGTGCACG                |                |
| 1451 | GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTGC<br>CGCATTGGTG GTGTGGCGG CGCGAATTAC GCGGCATGT CCCGCGCACG                 |                |

1501 TAGACTAGTG TTTAAACCGG ACCGGGGGG GGCTTAAGTG GGCTGCAAAA  
 ATCTGATCAC AAATTGGCC TGGCCCCCCC CCGAATTAC ACAGCTTTT  
  
 1551 CAAAACGGCC TCCTGTCAAG AAGCCGCTT TATCGGGTAG CCTCACTGCC  
 GTTTGCCGG AGGACAGTCC TTCGGCGAAA ATAGCCCATC GGAGTGACGG  
  
 1601 CGCTTCAG TCGGGAAACC TGCGTGCCA GCTGCATCAG TGAATCGGCC  
 GCGAAAGGTC AGCCCTTGG ACAGCACGGT CGACGTAGTC ACTTAGCCGG  
  
 1651 AACCGCGCGG GAGAGGCGGT TTGCGTATTG GGAGCCAGGG TGGTTTTCT  
 TTGCGCGCCC CTCTCCGCCA AACGCATAAC CCTCGGTCCC ACCAAAAAGA  
  
 1701 TTTCACCAAGT GAGACGGGCA ACAGCTGATT GCCCTTCACC GCCTGGCCCT  
 AAAGTGGTCA CTCTGCCCGT TGTCGACTAA CGGGAAAGTGG CGGACCGGGG  
  
 1751 GAGAGAGTTG CAGCAAGCGG TCCACGCTGG TTTGCCAG CAGGCGAAAAA  
 CTCTCTAAC CTCGTTGCCA AGGTGCGACC AACACGGGTC GTCCGCTTTT  
  
 1801 TCCTGTTGA TGGTGGTCAG CGGCAGGATA TAACATGAGC TGTCTCGGT  
 AGGACAAACT ACCACCAGTC GCCGCCCTAT ATTGTACTCG ACAGGAGCCA  
  
 1851 ATCGTCGTAT CCCACTACCG AGATGTCCGC ACCAACGCGC AGCCCGGACT  
 TAGCAGCATA GGGTGATGGC TCTACAGGCG TGGTTGCGCG TCAGGGCCTGA  
  
 1901 CGGTAATGGC ACGCATTGCG CCCAGCGCCA TCTGATCGTT GGCAACCAGC  
 GCCATTACCG TGCGTAACGC GGGTCGCGGT AGACTAGCAA CCGTTGGTCG  
  
 1951 ATCGCAGTGG GAACGATGCC CTCATTCAAGC ATTTGCATGG TTTGTTGAAA  
 TAGCGTCACC CTTGCTACGG GAGTAAGTCG TAAACGTACC AAACAACCTT  
  
 2001 ACCGGACATG GCACTCCAGT CGCCTTCCCG TTCCGCTATC GGCTGAATTT  
 TGGCCTGTAC CGTGAGGTCA GCGGAAGGGC AAGGCGATAG CCGACTTAAA  
  
 2051 GATTGCGAGT GAGATATTG TGCCAGCCAG CCAGACGCAG ACGCGCCGAG  
 CTAACGCTCA CTCTATAAAT ACGGTCGGTC GGTCTGCGTC TGCGCGGCTC  
  
 2101 ACAGAACTTA ATGGGCCAGC TAACAGCGCG ATTTGCTGGT GGCCCAATGC  
 TGTCTTGAAT TACCCGGTCG ATTGTGCGCG TAAACGACCA CCGGGTTACG  
  
 2151 GACCAGATGC TCCACGCCA GTCGCGTACC GTCCTCATGG GAGAAAATAA  
 CTGGTCTACG AGGTGCGGGT CAGCGCATGG CAGGAGTACC CTCTTTATT  
  
 2201 TACTGTTGAT GGGTGTCTGG TCAGAGACAT CAAGAAATAA CGCCGGAACA  
 ATGACAACTA CCCACAGACC AGTCTCTGTA GTTCTTTATT GCAGGCCTTGT  
  
 2251 TTAGTGCAGG CAGCTTCCAC AGCAATAGCA TCCTGGTCAT CCAGCGGATA  
 AATCACGTCC GTCGAAGGTG TCGTTATCGT AGGACCAGTA GGTGCGCTAT

ApalI

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2301 GTTAATAATC AGCCCACTGA CACGTTGCGC GAGAAGATTG TGCACCGCCG
 CAATTATTAG TCGGGTGACT GTGCAACGCG CTCTCTAAC ACGTGGCGGC

 2351 CTTTACAGGC TTGACGCCG CTTCGTTCTA CCATCGACAC GACCACGCTG
 GAAATGTCCG AAGCTGCGGC GAAGCAAGAT GGTAGCTGTG CTGGTGCGAC

2401 GCACCCAGTT GATCGGGCGCG AGATTAAATC GCCGGCACAA TTTGCGACGG
 CGTGGGTCAA CTAGCCGCGC TCTAAATTAG CGGCGCTGTT AAACGCTGCC

 2451 CGCGTGCAGG GCCAGACTGG AGGTGGCAAC GCCAATCAGC AACGACTGTT
 GCGCACGTCC CGGTCTGACC TCCACCGTTG CGGTTAGTCG TTGCTGACAA

 2501 TGCCCCGCCAG TTGTTGTGCC ACGCGGTTAG GAATGTAATT CAGCTCCGCC
 ACGGGCGGTC AACAAACACGG TGCGCCAATC CTTACATTAA GTCGAGGCAG

 2551 ATCGCCGCTT CCACTTTTTC CCGCGTTTTC GCAGAAACGT GGCTGGCCTG
 TAGCGGCGAA GGTAAAAAAG GGCGAAAAG CGTCTTGCA CCGACCGGAC

 2601 GTTCACCACG CGGGAAACGG TCTGATAAGA GACACCGGCA TACTCTGCAG
 CAAGTGGTGC GCCCTTGCC AGACTATTCT CTGTGGCCGT ATGAGACGCT

 2651 CATCGTATAA CGTTACTGGT TTCACATTCA CCACCCGTAA TTGACTCTCT
 GTAGCATATT GCAATGACCA AAGTGTAAAGT GGTGGGACTT AACTGAGAGA

 2701 TCCGGGCGCT ATCATGCCAT ACCCGGAAAG GTTTTGCAGCC ATTGATGCT
 AGGCCCGCGA TAGTACGGTA TGGCGTTTTC CAAAACGGG TAAGCTACGA

 2751 AGCCATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG
 TCGGTACACT CGTTTCCGG TCGTTTCCG GTCCTGGCA TTTTCCGGC

 2801 CGTTGCTGGC GTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
 GCAACGACCG CAAAAAGGTA TCCGAGGCCGG GGGGACTGCT CGTAGTGTGTT

 2851 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
 TTAGCTGCAG GTTCAGTCTC CACCGCTTTG GGCTGTCCCTG ATATTTCTAT

 2901 CCAGGCGTTT CCCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
 GGTCCGCAAA GGGGGACCTT CGAGGGAGCA CGCGAGAGGA CAAGGCTGGG

 2951 TGCCGCTTAC CGGATAACCTG TCCGCTTTTC TCCCTCGGG AAGCGTGGCG
 ACGGCGAATG GCCTATGGAC AGGCGGAAAG AGGGAAGCCC TTCGCACCGC

 3001 CTTTCTCATA GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTG
 GAAAGAGTAT CGAGTGCAC ATCCATAGAG TCAAGCCACA TCCAGCAAGC

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3051 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCC  
 GAGGTTCGAC CCGACACACG TGCTGGGGG GCAAGTCGGG CTGGCGACGC  
  
 3101 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA  
 GGAATAGGCC ATTGATAGCA GAACTCAGGT TGGGCCATTG TGTGCTGAAT  
  
 3151 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT  
 AGCGGTGACC GTCGTCGGTG ACCATTGTCC TAATCGTCTC GCTCCATACA  
  
 3201 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA  
 TCCGCCACGA TGTCTCAAGA ACTTCACCAAC CGGATTGATG CCGATGTGAT  
  
 3251 GAAGAACAGT ATTTGGTATC TGCGCTCTGC TGTAGCCAGT TACCTCGGA  
 CTTCTGTCA TAAACCATAG ACGCGAGACG ACATCGGTCA ATGGAAGCCT

3301 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG  
 TTTTCTCAAC CATCGAGAAC TAGGCCGTTT GTTTGGTGGC GACCATCGCC  
  
 3351 TGGTTTTTTT GTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC  
 ACCAAAAAAA CAAACGTTCG TCGTCTAATG CGCGTCTTTT TTTCCTAGAG  
  
 3401 AAGAAGATCC TTTGATCTTT TCTACGGGT CTGACGCTCA GTGGAACGAA  
 TTCTTCTAGG AAACTAGAAA AGATGCCCA GACTGCGAGT CACCTTGCTT  
  
 3451 AACTCACGTT AAGGGATTTT GGTCAGATCT AGCACCAGGC GTTTAAGGGC  
 TTGAGTGCAA TTCCCTAAAA CCAGTCTAGA TCGTGGTCCG CAAATTCCCG  
  
 3501 ACCAATAACT GCCTTAAAAA AATTACGCC CGCCCTGCCA CTCATCGCAG  
 TGGTTATTGA CGGAATTTTT TTAATGCGGG GCAGGACGGT GAGTAGCGTC  
  
 3551 TACTGTTGTA ATTCAATTAG CATTCTGCCG ACATGGAAGC CATCACAAAC  
 ATGACAACAT TAAGTAATTG GTAAGACGGC TGTACCTTCG GTAGTGTGG  
  
 3601 GGCATGATGA ACCTGAATCG CCAGCGGCAT CAGCACCTTG TCGCCTTGCG  
 CCGTACTACT TGGACTTAGC GGTCCCGTA GTCGTGGAAC AGCGAACGCG  
  
 3651 TATAATATTG GCCCATAGTG AAAACGGGGG CGAAGAAGTT GTCCATATTG  
 ATATTATAAA CGGGTATCAC TTTTGCCCCC GCTTCTTCAA CAGGTATAAC  
  
 3701 GCTACGTTA AATCAAAACT GGTGAAACTC ACCCAGGGAT TGGCTGAGAC  
 CGATGCAAAT TTAGTTTGA CCACTTGAG TGGGTCCCTA ACCGACTCTG  
  
 3751 GAAAAACATA TTCTCAATAA ACCCTTTAGG GAAATAGGCC AGGTTTCAC  
 CTTTTGTAT AAGAGTTATT TGGGAAATCC CTTTATCCGG TCCAAAAGTG  
  
 3801 CGTAACACGG CACATCTTGC GAATATATGT GTAGAAACTG CCGGAAATCG  
 GCATTGTGCG GTGTAGAACG CTTATATACA CATCTTGAC GGCCTTCTG  
  
 3851 TCGTGGTATT CACTCCAGAG CGATGAAAAC GTTTCAGTTT GCTCATGGAA  
 AGCACCATAA GTGAGGTCTC GCTACTTTG CAAAGTCAAA CGAGTACCTT  
  
 3901 AACGGTGTAA CAAGGGTGAA CACTATCCCA TATCACCAGC TCACCGTCTT  
 TTGCCACATT GTTCCCACTT GTGATAGGGT ATAGTGGTCG AGTGGCAGAA  
  
 3951 TCATTGCCAT ACGGAACCTCC GGGTGAGCAT TCATCAGGCG GGCAAGAATG  
 AGTAACGGTA TGCCTTGAGG CCCACTCGTA AGTAGTCCGC CCGTTCTTAC  
  
 4001 TGAATAAAGG CCGGATAAAA CTTGTGCTTA TTTTCTTTA CGGTCTTTAA  
 ACTTATTTCG GGCCTATTAA GAACACGAAT AAAAAGAAAT GCCAGAAATT  
  
 4051 AAAGGCCGTA ATATCCAGCT GAACGGTCTG GTTATAGGTA CATTGAGCAA  
 TTTCCGGCAT TATAGGTGCA CTTGCCAGAC CAATATCCAT GTAACCGTCTT  
  
 4101 CTGACTGAAA TGCCCTAAAA TGTTCTTTAC GATGCCATTG GGATATATCA  
 GACTGACTTT ACGGAGTTT ACAAGAAATG CTACGGTAAC CCTATATAGT  
  
 4151 ACGGTGGTAT ATCCAGTGAT TTTTTCTCC ATTTTAGCTT CCTTAGCTCC  
 TGCCACCATA TAGGTCACTA AAAAAAGAGG TAAAATCGAA GGAATCGAGG  
  
 4201 TGAAAATCTC GATAACTCAA AAAATACGCC CGGTAGTGAT CTTATTCAT  
 ACTTTTAGAG CTATTGAGTT TTTTATGCGG GCCATCACTA GAATAAAGTA

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4251 TATGGTGAAA GTTGGAACCT CACCCGACGT CTAATGTGAG TTAGCTCACT  
ATACCACTTT CAACCTTGGA GTGGGCTGCA GATTACACTC AATCGAGTGA

4301 CATTAGGCAC CCCAGGCTTT ACACHTTATG CTTCCGGCTC GTATGTTGTG  
GTAATCCGTG GGGTCCGAAA TGTGAAATAC GAAGGCCGAG CATAACAACAC

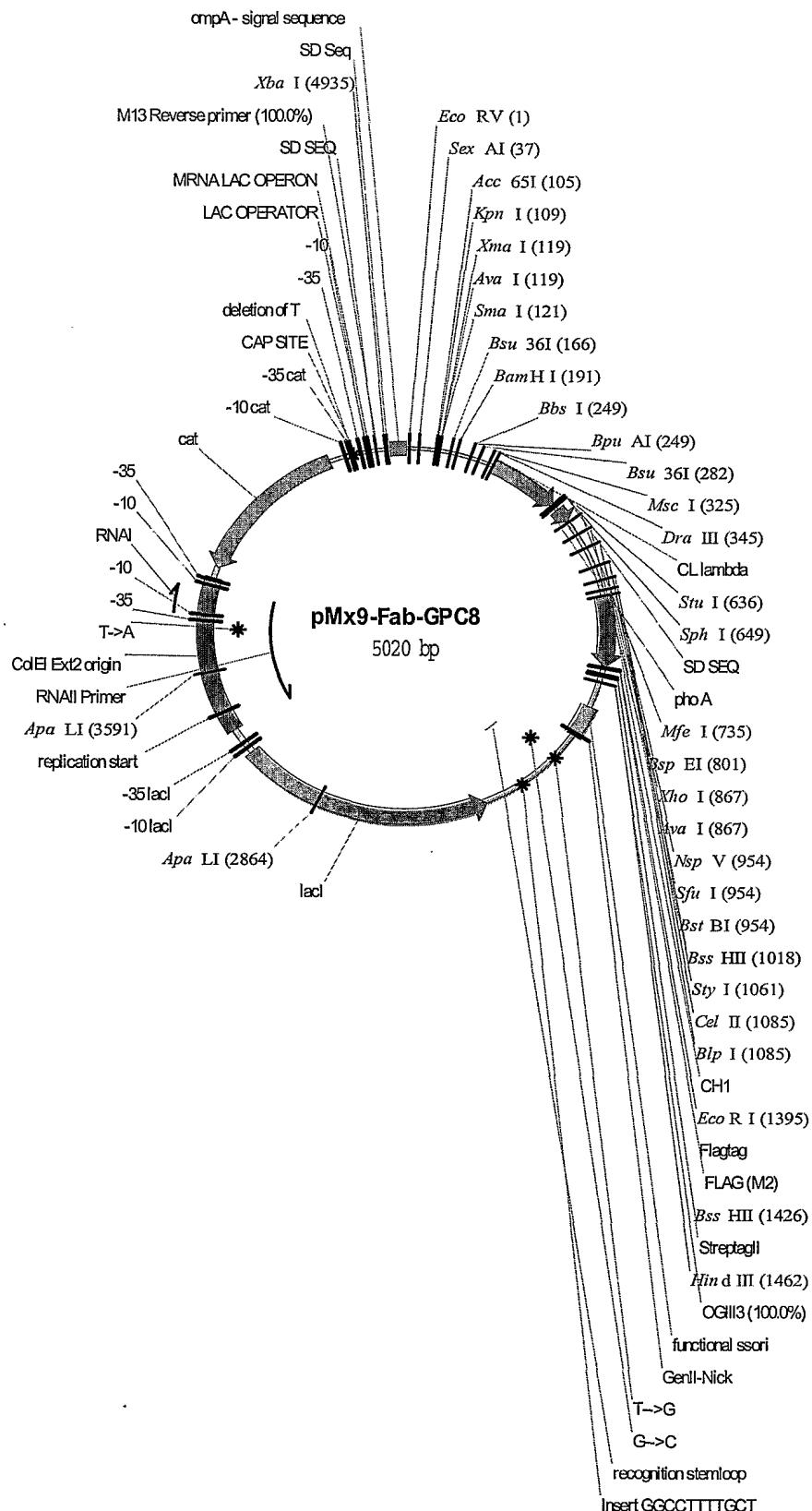
M13 Reverse primer 100.0%

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4351 TGGAATTGTG AGCGGATAAC AATTCACAC AGGAAACAGC TATGACCATG  
ACCTAACAC TCGCCTATTG TTAAAGTGTG TCCTTGTG ATACTGGTAC

4401 ATTACGAATT  
TAATGCTTAA

# Figure 13



## Figure 13 (cont)

|                                                                                                                                                        |                                      |
|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------|
| <p>EcoRV</p> <hr/> <pre> ~~~  1  ATCGTGCTGA CCCAGCCGCC TTCAGTGAGT GGCGCACCAAG GTCAGCGTGT TAGCACGACT GGGTCGGCGG AAGTCACTCA CCGCGTGGTC CAGTCGCACA </pre> | <p>SexAI</p> <hr/> <pre> ~~~~~</pre> |
| <pre> 51  GACCATCTCG TGTAGCGGCAGA GCAGCAGCAA CATTGGCAGC AACTATGTGA CTGGTAGAGC ACATCGCCGT CGTCGTCGTT GTAACCGTCG TTGATAACACT </pre>                      |                                      |
| <p>XmaI</p> <hr/>                                                                                                                                      |                                      |
| <p>KpnI</p> <hr/> <pre> ~~~~~</pre>                                                                                                                    | <p>SmaI</p> <hr/> <pre> ~~~~~</pre>  |
| <p>Acc65I</p> <hr/> <pre> ~~~~~</pre>                                                                                                                  | <p>AvaI</p> <hr/> <pre> ~~~~~</pre>  |
| <pre> 101 GCTGGTACCA GCAGTTGCCCGGGACGGCGCG CGAAACTGCT GATTTATGAT CGACCATGGT CGTCAACGGG CCCTGCCGCG GCTTGACGA CTAAATACTA </pre>                          |                                      |
| <p>Bsu36I</p> <hr/>                                                                                                                                    |                                      |
| <pre> 151 AACAAACCAGC GTCCCTCAGG CGTGCCGGAT CGTTTAGCG GATCCAAAAG TTGTTGGTCC CAGGGAGTCC GCACGGCCTA GCAAAATCGC CTAGGTTTC </pre>                          | <p>BamHI</p> <hr/> <pre> ~~~~~</pre> |
| <p>BpuAI</p> <hr/>                                                                                                                                     |                                      |
| <p>BbsI</p> <hr/> <pre> ~~~~~</pre>                                                                                                                    |                                      |
| <pre> 201 CGGCACCAGC GCGAGCCTTG CGATTACGGG CCTGCAAAGC GAAGACGAAG GCCGTGGTCC CGCTCGAAC GCTAATGCCG GGACGTTTC CTTCTGCTTC </pre>                           |                                      |
| <p>Bsu36I</p> <hr/>                                                                                                                                    |                                      |
| <pre> 251 CGGATTATTA TTGCCAGAGC TATGACATGC CTCAGGCTGT GTTGGCGGC GCCTAATAAT AACGGTCTCG ATACTGTACG GAGTCCGACA CAAACCGCCG </pre>                          |                                      |
| <p>MscI</p> <hr/>                                                                                                                                      |                                      |
| <p>DraIII</p> <hr/>                                                                                                                                    |                                      |
| <pre> 301 GGCACGAAGT TTAACCGTTC TTGGCCAGCC GAAAGCCGCA CCGAGTGTGA CCGTGCTTCA AATTGGCAAG AACCGGTCGG CTTTCGGCGT GGCTCACACT </pre>                         | <pre> ~~~~~</pre>                    |
| <pre> 351 CGCTGTTTCC GCCGAGCAGC GAAGAATTGC AGGCGAACAA AGCGACCCCTG GCGACAAAGG CGGCTCGTCG CTTCTTAACG TCCGCTTGTT TCGCTGGGAC </pre>                        | <pre> ~~~~~</pre>                    |
| <pre> 401 GTGTGCCTGA TTAGCGACTT TTATCCGGGA GCCGTGACAG TGGCCTGGAA CACACGGACT AATCGCTGAA AATAGGCCCT CGGCACTGTC ACCGGACCTT </pre>                         | <pre> ~~~~~</pre>                    |
| <pre> 451 GGCAGATAGC AGCCCCGTCA AGGCAGGGAGT GGAGACCACC ACACCCTCCA CCGTCTATCG TCGGGGCAGT TCCGCCCTCA CCTCTGGTGG TGTGGGAGGT </pre>                        | <pre> ~~~~~</pre>                    |
| <pre> 501 AACAAAGCAA CAACAAGTAC GCGGCCAGCA GCTATCTGAG CCTGACGCCT TTGTTTCGTT GTTGTTCATG CGCCGGTCGT CGATAGACTC GGACTGCGGA </pre>                         | <pre> ~~~~~</pre>                    |
| <pre> 551 GAGCAGTGGAA AGTCCCACAG AAGCTACAGC TGCCAGGTCA CGCATGAGGG CTCGTCACCT TCAGGGTGTGTC TTCGATGTGTC ACGGTCCAGT GCGTACTCCC </pre>                     | <pre> ~~~~~</pre>                    |

601 GAGCACCGTG GAAAAAACCG TTGCGCCGAC TGAGGCCTGA TAAGCATGCG  
CTCGTGGCAC CTTTTTGGC AACCGGGCTG ACTCCGGACT ATTCGTACGC

651 TAGGAGAAAA TAAAATGAAA CAAAGCACTA TTGCACTGGC ACTCTTACCG  
ATCCTCTTTT ATTTTACTTT GTTTCGTGAT AACGTGACCG TGAGAATGGC

## MfeI

701 TTGCTCTTCA CCCCTGTTAC CAAAGCCCAG GTGCAATTGA AAGAAAGCGG  
AACGAGAAGT GGGGACAATG GTTTCGGTC CACGTTAACT TTCTTCGCC

## BspEI

751 CCCGGCCCTG GTGAAACCGA CCCAAACCCT GACCCTGACC TGTACCTTT  
GGGCCGGGAC CACTTGGCT GGGTTGGGA CTGGGACTGG ACATGGAAAA

## BspEI

801 CCGGATTTAG CCTGTCCACG TCTGGCGTTG GCGTGGGCTG GATTGCCAG  
GCCCTAAATC GGACAGGTGC AGACCGAAC CGCACCCGAC CTAAGCGGTC

## XbaI

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AvaI

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851 CCGCCTGGGA AAGCCCTCGA GTGGCTGGCT CTGATTGATT GGGATGATGA  
GGCGGACCCT TTCGGGAGCT CACCGACCGA GACTAACTAA CCCTACTACT

901 TAAGTATTAT AGCACCAAGCC TGAAAACGCG TCTGACCATT AGCAAAGATA  
ATTCTATAATA TCGTGGTCGG ACTTTGCGC AGACTGGTAA TCGTTTCTAT

## BstBI

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SfuI

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## NspV

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951 CTTCGAAAAA TCAGGTGGTG CTGACTATGA CCAACATGGA CCCGGTGGAT
GAAGCTTTT AGTCCACAC GACTGATACT GGTTGTACCT GGGCCACCTA

BssHII

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1001 ACGGCCACCT ATTATTGCGC GCGTTCTCCT CGTTATCGTG GTGCTTTGA  
TGCCGGTGGGA TAATAACGCG CGCAAGAGGA GCAATAGCAC CACGAAAAC

## BpuI

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StyI

CclII

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1051 TTATTGGGGC CAAGGCACCC TGGTGACGGT TAGCTCAGCG TCGACCAAAG  
AATAACCCCCG GTTCCGTGGG ACCACTGCCA ATCGAGTCGC AGCTGGTTTC

1101 GTCCAAGCGT GTTTCCGCTG GCTCCGAGCA GCAAAAGCAC CAGCGGGCGC  
CAGGTTCGCA CAAAGGCGAC CGAGGCTCGT CGTTTCGTG GTCGCCGCCG

1151 ACGGCTGCC C TGGGCTGCCT GGTTAAAGAT TATTTCCCGG AACCAAGTCAC

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TGCCGACGGG ACCCGACGGA CCAATTCTA ATAAAGGGCC TTGGTCAGTG  
 1201 CGTGAGCTGG AACAGCGGGG CGCTGACCAG CGGCGTGCAT ACCTTCGG  
 GCACTCGACC TTGTGCCCCC GCGACTGGTC GCCGCACGTA TGGAAAGGCC  
 1251 CGGTGCTGCA AAGCAGCGGC CTGTATAGCC TGAGCAGCGT TGTGACCGTG  
 GCCACGACGT TTCGTCGCCG GACATATCGG ACTCGTCGCA ACACTGGCAC  
 1301 CCGAGCAGCA GCTTAGGCAC TCAGACCTAT ATTTGCAACG TGAACCATAA  
 GGCTCGTCGT CGAATCCGT AGTCTGGATA TAAACGTTGC ACTTGGTATT

EcoRI

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1351 ACCGAGAAC ACCAAAGTGG ATAAAAAAAGT GGAACCGAAA AGCGAATTG
 TGGCTCGTTG TGGTTTCACC TATTTTTCA CCTTGGCTTT TCGCTTAAGC

BssHII

~~~~~

1401 ACTATAAAGA TGACGGATGAC AAAGGGCGCGC CGTGGAGCCA CCCGCAGTTT  
 TGATATTTCT ACTGCTACTG TTTCCGCGCG GCACCTCGGT GGGCGTCAAA

HindIII

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1451 GAAAAATGAT AAGCTTGACC TGTGAAGTGA AAAATGGCGC AGATTGTGCG
 CTTTTACTA TTCGAACTGG ACACTTCACT TTTTACCGCG TCTAACACGC
 OGIII3 100.0%

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1501 ACATTTTTT TGTCTGCCGT TTAATTAAAG GGGGGGGGGG GCCGGCCTGG
 TGTAAAAAAA ACAGACGGCA AATTAATTTC CCCCCCCCCC CGGCCGGACC

1551 GGGGGGGTGT ACATGAAATT GTAAACGTTA ATATTTGTT AAAATTGCG
 CCCCCCCCACA TGTACTTTAA CATTGCAAT TATAAAACAA TTTTAAGCGC

1601 TTAAATTTT GTTAAATCAG CTCATTTTT AACCAATAGG CCGAAATCGG
 AATTAAAAAA CAATTAGTC GAGTAAAAAA TTGGTTATCC GGCTTAGGCC

1651 CAAAATCCCT TATAATCAA AAGAATAGAC CGAGATAGGG TTGAGTGGT
 GTTTAGGGA ATATTTAGTT TTCTTATCTG GCTCTATCCC AACTCACAAAC

1701 TTCCAGTTG GAACAAGAGT CCACTATTAA AGAACGTGGA CTCCAACGTC
 AAGGTCAAAC CTTGTTCTCA GGTGATAATT TCTTGCACCT GAGGTTGCAG

1751 AAAGGGCGAA AAACCGTCTA TCAGGGCGAT GGCCCAC TAC GAGAACCATC
 TTTCCCGCTT TTTGGCAGAT AGTCCCGCTA CCGGGTGATG CTCTTGGTAG

1801 ACCCTAATCA AGTTTTTGG GGTGAGGTG CCGTAAAGCA CTAAATCGGA
 TGGGATTAGT TCAAAAAACC CCAGCTCCAC GGCATTCGT GATTTAGCCT

1851 ACCCTAAAGG GAGCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC
 TGGGATTTCCTC CTCGGGGGCT AAATCTCGAA CTGCCCCCTT CGGCCGCTTG

1901 GTGGCGAGAA AGGAAGGGAA GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT
 CACCGCTCTT TCCTTCCCTT CTTTCGCTTT CCTCGCCCGC GATCCCACGA

1951 GGCAAGTGT A GCGGTACGC TGCGCGTAAC CACCAACACCC GCCGCGCTTA
 CCGTTCACAT CGCCAGTGC GCGCGATTG GTGGTGTGGG CGGCCGCGAAT

2001 ATGCGCCGCT ACAGGGCGCG TGCTAGACTA GTGTTAAAC CGGACCGGGG
 TACCGGGCGA TGTCCCGCGC ACGATCTGAT CACAAATTG GCCTGGCCCC

 2051 GGGGGCTTAA GTGGGCTGCA AAACAAAACG GCCTCCTGTC AGGAAGCCGC
 CCCCCGAATT CACCCGACGT TTTGTTTGC CGGAGGACAG TCCTTCGGCG

 2101 TTTTATCGGG TAGCCTCACT GCCCGCTTTC CAGTCGGAA ACCTGTCGTG
 AAAATAGCCC ATCGGAGTGA CGGGCGAAAG GTCAGCCCTT TGGACAGCAC

 2151 CCAGCTGCAT CAGTGAATCG GCCAACGCGC GGGGAGAGGC GGTTTGCATA
 GTCGACGTA GTCACTTAGC CGGTTGCGCG CCCCTCTCCG CCAAACGCAT

 2201 TTGGGAGCCA GGGTGGTTT TCTTTCAACC AGTGAGACGG GCAACAGCTG
 AACCTCGGT CCCACCAAAA AGAAAAGTGG TCACTCTGCC CGTTGTCGAC

 2251 ATTGCCCTTC ACCGCCTGGC CCTGAGAGAG TTGCAGCAAG CGGTCCACGC
 TAACGGGAAG TGGCGGACCG GGAECTCTCTC AACGTCGTTG GCCAGGTGCG

 2301 TGTTTGCCC CAGCAGGCGA AAATCCTGTT TGATGGTGGT CAGCGGCGGG
 ACCAAACGGG GTCGTCGCT TTTAGGACAA ACTACCACCA GTCGCCGCC

 2351 ATATAAACATG AGCTGTCCTC GGTATCGTCG TATCCCACTA CCGAGATGTC
 TATATTGTAC TCGACAGGAG CCATAGCAGC ATAGGGTGAT GGCTCTACAG

 2401 CGCACCAACG CGCAGCCCGG ACTCGGTAAT GGCACGCATT GCGCCCAGCG
 GCGTGGTTGC GCGTCGGGCC TGAGCCATTA CGTGCGTAA CGCGGGTCGC

 2451 CCATCTGATC GTTGGCAACC AGCATCGCAG TGGGAACGAT GCCCTCATTC
 GGTAGACTAG CAACCGTTGG TCGTAGCGTC ACCCTTGCTA CGGGAGTAAG

 2501 AGCATTGCA TGGTTGTTG AAAACCGGAC ATGGCACTCC AGTCGCCTTC
 TCGTAAACGT ACCAAACAAC TTTTGGCCTG TACCGTGAGG TCAGCGGAAG

 2551 CCGTTCCGCT ATCGGCTGAA TTTGATTGCG AGTGAGATAT TTATGCCAGC
 GGCAAGGCAGA TAGCCGACTT AAACTAACGC TCACTCTATA AATACGGTCG

 2601 CAGCCAGACG CAGACGCGCC GAGACAGAAC TTAATGGGCC AGCTAACAGC
 GTCGGTCTGC GTCTGCGCGG CTCTGTCTTG AATTACCCGG TCGATTGTCG

 2651 GCGATTGCT GGTGGCCCAA TGCGACCAGA TGCTCCACGC CCAGTCGCGT
 CGCTAAACGA CCACCGGGTT ACGCTGGTCT ACGAGGTGCG GGTCAAGCGCA

 2701 ACCGTCCCTCA TGGGAGAAAA TAATACTGTT GATGGGTGTC TGGTCAGAGA
 TGGCAGGAGT ACCCTCTTT ATTATGACAA CTACCCACAG ACCAGTCTCT

 2751 CATCAAGAAA TAACGCCGGA ACATTAGTGC AGGCAGCTTC CACAGCAATA
 GTAGTTCTTT ATTGCGGCCT TGTAAATCACG TCCGTGAAAG GTGTCGTTAT

 2801 GCATCCTGGT CATCCAGCGG ATAGTTAATA ATCAGCCCAC TGACACGTTG
 CGTAGGACCA GTAGGTCGCC TATCAATTAT TAGTCGGGTG ACTGTGCAAC

ApaLI

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2851 CGCGAGAAGA TTGTGCACCG CCGCTTTACA GGCTTCGACG CCGCTTCGTT  
 GCGCTCTTCT AACACGTGGC GGCGAAATGT CCGAAGCTGC GGCGAAGCAA

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2901 CTACCATCGA CACGACCACG CTGGCACCCA GTTGATCGGC GCGAGATTAA  
GATGGTAGCT GTGCTGGTGC GACCGTGGGT CAACTAGCCG CGCTCTAAAT

2951 ATCGCCGCGA CAATTGCGA CGGCGCGTGC AGGGCCAGAC TGGAGGTGGC  
TAGCGCGCT GTTAAACGCT GCCGCGCACG TCCC GGTCCTG ACCTCCACCG

3001 AACGCCAATC AGCAACGACT GTTTGCCGC CAGTTGTTGT GCCACGCGGT  
TTGCGGTTAG TCGTTGCTGA CAAACGGGCG GTCAACAACA CGGTGCGCCA

3051 TAGGAATGTA ATTCA GCTCC GCCATCGCCG CTTCCACTTT TTCCCGCGTT  
ATCCTTACAT TAAGTCGAGG CGGTAGCGGC GAAGGTGAAA AAGGGCGCAA

3101 TTCCGAGAAA CGTGGCTGGC CTGGTTCAAC ACGCGGGAAA CGGTCTGATA  
AACCGTCTTT GCACCGACCG GACCAAGTGG TGCGCCCTTT GCCAGACTAT

3151 AGAGACACCG GCATACTCTG CGACATCGTA TAACGTTACT GGTTTCACAT  
TCTCTGTGGC CGTATGAGAC GCTGTAGCAT ATTGCAATGA CCAAAGTGTA

3201 TCACCA CACCT GAATTGACTC TCTTCCGGGC GCTATCATGC CATAACCGCGA  
AGTGGTGGGA CTTAACTGAG AGAAGGCCCG CGATAGTACG GTATGGCGCT

3251 AAGGTTTGC GCCATTGAT GCTAGCCATG TGAGCAAAAG GCCAGCAAAA  
TTCCAAAACG CGGTAAGCTA CGATCGGTAC ACTCGTTTC CGGTCGTTTT

3301 GGCCAGGAAC CGTAAAAGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC  
CCGGTCCTTG GCATTTTCC GGCGAACCGA CCGCAAAAG GTATCCGAGG

3351 GCCCCCCTGA CGAGCATCAC AAAATCGAC GCTCAAGTCA GAGGTGGCGA  
CGGGGGGACT GCTCGTAGTG TTTTAGCTG CGAGTTCACT CTCCACCGCT

3401 AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT  
TTGGGCTGTC CTGATATTTC TATGGTCCGC AAAGGGGGAC CTTCGAGGGA

3451 CGTGCCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT  
GCACCGCGA GGACAAGGCT GGGACGGCGA ATGGCCTATG GACAGGGCGA

3501 TTCTCCCTTC GGGAAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT  
AAGAGGGAAAG CCCTCGCAC CGCGAAAGAG TATCGAGTGC GACATCCATA

ApalI

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3551 CTCAGTTCGG TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC
GAGTCAAGCC ACATCCAGCA AGCGAGGTTC GACCCGACAC ACGTGCTTGG

3601 CCCCGTTCAAG CCCGACCGCT GCGCCTTATC CGGTAACAT CGTCTTGAGT
GGGGCAAGTC GGGCTGGCGA CGCGGAATAG GCCATTGATA GCAGAACTCA

3651 CCAACCCGGT AAGACACGAC TTATGCCAC TGGCAGCAGC CACTGGTAAC
GGTGGGCCA TTCTGTGCTG AATAGCGGTG ACCGTGTCG GTGACCATTG

3701 AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG
TCCTAATCGT CTCGCTCCAT ACATCCGCCA CGATGTCTCA AGAACTTCAC

3751 GTGGCCTAAC TACGGCTACA CTAGAAGAAC AGTATTGGT ATCTGCCTC
CACCGGATTG ATGCCGATGT GATCTTCTTG TCATAAACCA TAGACGCGAG

3801 TGCTGTAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC

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ACGACATCGG TCAATGGAAG CCTTTTCTC AACCATCGAG AACTAGGCCG
 3851 AAACAAACCA CCGCTGGTAG CGGTGGTTT TTTGTTGCA AGCAGCAGAT
 TTTGTTGGT GGCGACCATC GCCACCAAAA AAACAAACGT TCGTCGTCTA
 3901 TACGCGCAGA AAAAAAGGAT CTCAAGAAGA TCCTTGATC TTTTCTACGG
 ATGCGCGTCT TTTTTCTA GAGTTCTTCT AGGAAACTAG AAAAGATGCC
 3951 GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCAGA
 CCAGACTGCG AGTCACCTG CTTTGAGTG CAATTCCCTA AAACCAGTCT
 4001 TCTAGCACCA GGCCTTAAG GGCACCAATA ACTGCCCTAA AAAAATTACG
 AGATCGTGGT CCGCAAATTC CCGTGGTTAT TGACGGAATT TTTTTAATGCA
 4051 CCCCCCCCTG CCACTCATCG CAGTACTGTT GTAATTCTATT AAGCATTCTG
 GGGGCGGGAC GGTGAGTAGC GTCATGACAA CATTAAGTAA TTCGTAAGAC
 4101 CCGACATGGA AGCCATCACA AACGGCATGA TGAACCTGAA TCGCCAGCGG
 GGCTGTACCT TCGGTAGTGT TTGCCGTACT ACTTGGACTT AGCGGTCGCC
 4151 CATCAGCACC TTGTCGCCTT GCGTATAATA TTTGCCATA GTGAAAACGG
 GTAGTCGTGG AACAGCGGAA CGCATATTAT AAACGGGTAT CACTTTGCC
 4201 GGGCGAAGAA GTTGTCCATA TTGGCTACGT TTAAATCAAA ACTGGTGAAA
 CCCGCTTCTT CAACAGGTAT AACCGATGCA AATTTAGTT TGACCACCTT
 4251 CTCACCCAGG GATTGGCTGA GACGAAAAAC ATATTCTCAA TAAACCCCTT
 GAGTGGGTCC CTAACCGACT CTGCTTTTG TATAAGAGTT ATTTGGGAAA
 4301 AGGGAAATAG GCCAGGTTTT CACCGTAACA CGCCACATCT TGCGAATATA
 TCCCTTTATC CGGTCCAAAA GTGGCATTGT GCGGTGTAGA ACGCTTATAT
 4351 TGTGTAGAAA CTGCCGGAAA TCGTCGTGGT ATTCACTCCA GAGCGATGAA
 ACACATCTT GACGGCCTTT AGCAGCACCA TAAGTGAGGT CTCGCTACTT
 4401 AACGTTTCAG TTTGCTCATG GAAAACGGTG TAACAAGGGT GAACACTATC
 TTGCAAAGTC AAACGAGTAC CTTTGCCAC ATTGTTCCCA CTTGTGATAG
 4451 CCATATCACC AGCTCACCGT CTTTCATTGC CATACGGAAC TCCGGGTGAG
 GGTATAGTGG TCGAGTGGCA GAAAGTAACG GTATGCCCTG AGGCCCACTC
 4501 CATTCACTCAG GCGGGCAAGA ATGTGAATAA AGGCCGGATA AAACTTGTGC
 GTAAGTAGTC CGCCCGTTCT TACACTTATT TCCGGCCTAT TTTGAACACG
 4551 TTATTTTCT TTACGGTCTT TAAAAAGGCC GTAATATCCA GCTGAACGGT
 AATAAAAAGA AATGCCAGAA ATTTTCCGG CATTATAGGT CGACTTGCCA
 4601 CTGGTTATAG GTACATTGAG CAACTGACTG AAATGCCTCA AAATGTTCTT
 GACCAATATC CATGTAACTC GTTGACTGAC TTTACGGAGT TTTACAAGAA
 4651 TACGATGCCA TTGGGATATA TCAACGGTGG TATATCCAGT GATTTTTTC
 ATGCTACGGT AACCCCTATAT AGTTGCCACC ATATAGGTCA CTAAAAAAAG
 4701 TCCATTTAG CTTCCCTTAGC TCCTGAAAAT CTCGATAACT CAAAAAAATAC
 AGGTAAAATC GAAGGAATCG AGGACTTTA GAGCTATTGA GTTTTTATG
 4751 GCCCGGTAGT GATCTTATTT CATTATGGTG AAAGTTGGAA CCTCACCCGA

38/49

CGGGCCATCA CTAGAATAAA GTAATACCAC TTTCAACCTT GGAGTGGGCT

4801 CGTCTAATGT GAGTTAGCTC ACTCATTAGG CACCCCAGGC TTTACACTTT
GCAGATTACA CTCAATCGAG TGAGTAATCC GTGGGGTCCG AAATGTGAAA4851 ATGCTTCGG CTCGTATGTT GTGTGGAATT GTGAGCGGAT AACAAATTCA
TACGAAGGCC GAGCATAACAA CACACCTTAA CACTCGCCTA TTGTTAAAGT

M13 Reverse primer 100.0%

XbaI

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4901 CACAGGAAAC AGCTATGACC ATGATTACGA ATTTCTAGAT AACGAGGGCA  
GTGTCTTTG TCGATACTGG TACTAATGCT TAAAGATCTA TTGCTCCGT4951 AAAAATGAAA AAGACAGCTA TCGCGATTGC AGTGGCACTG GCTGGTTTCG  
TTTTTACTTT TTCTGTCGAT AGCGCTAACG TCACCGTGAC CGACCAAAGC

EcoRV

~~~

5001 CTACCGTAGC GCAGGCCGAT
GATGGCATCG CGTCCGGCTA

Figure 14

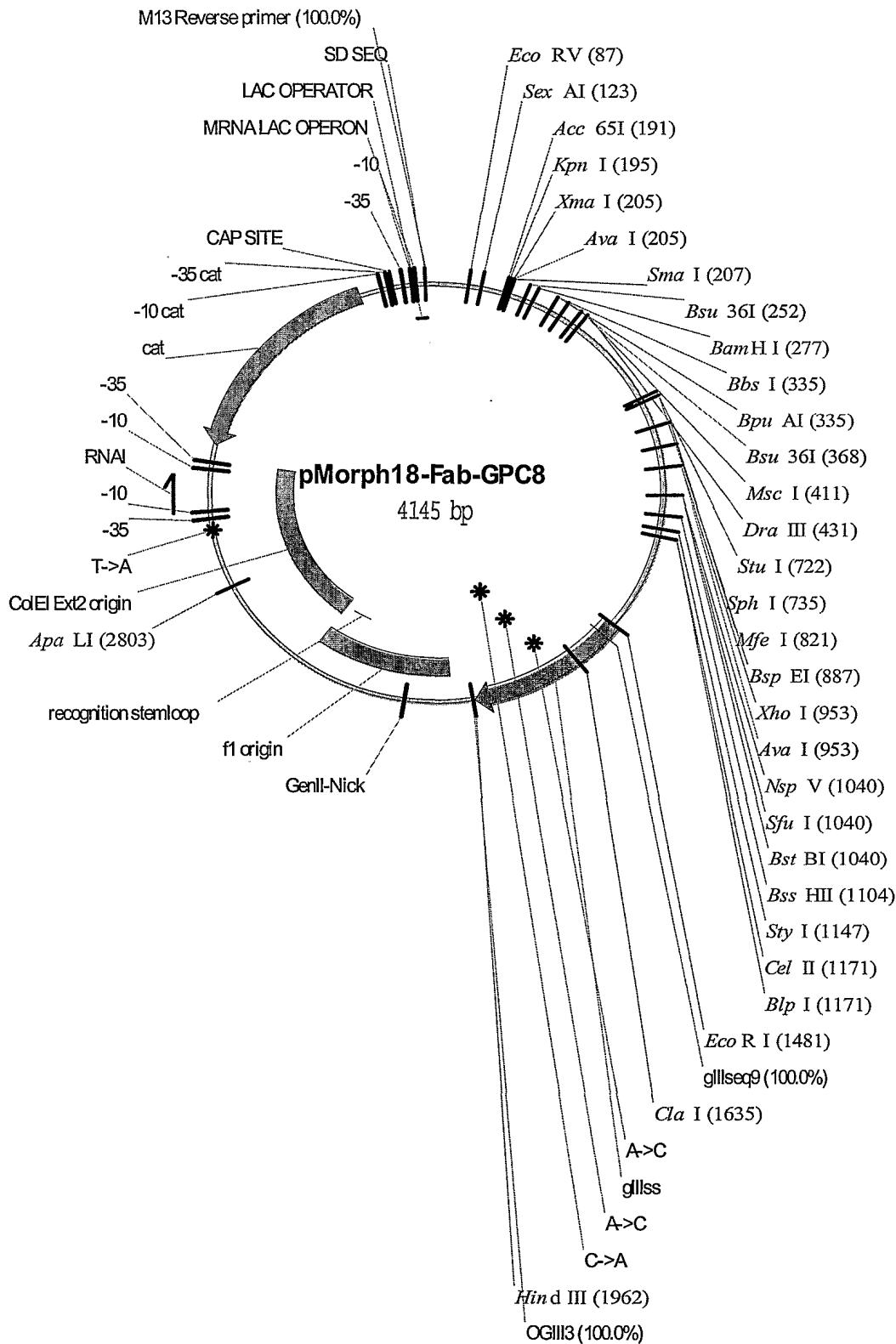


Figure 14 (cont)

1 TCAGATAACG AGGGCAAAAA ATGAAAAAGA CAGCTATCGC GATTGCAGTG
AGTCTATTGC TCCCGTTTT TACTTTTCT GTCGATAGCG CTAACGTCAC

EcoRV
~~~~~

51 GCACTGGCTG GTTCGCTAC CGTAGCGCAG GCCGATATCG TGCTGACCCA  
CGTGACCGAC CAAAGCGATG GCATCGCGTC CGGCTATAGC ACGACTGGGT

SexAI  
~~~~~

101 GCCGCCTTCA GTGAGTGGCG CACCAGGTCA GCGTGTGACC ATCTCGTGT
CGGCAGGAAGT CACTCACCGC GTGGTCCAGT CGCACACTGG TAGAGCACAT

KpnI
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151 GCGGCAGCAG CAGAACATT GGCAGCAACT ATGTGAGCTG GTACCAGCAG  
CGCCGTCGTC GTCGTTGTAA CCGTCGTTGA TACACTCGAC CATGGTCGTC

Acc65I  
~~~~~

201 TTGCCCGGGA CGGCCCGAA ACTGCTGATT TATGATAACA ACCAGCGTCC
AACGGGCCCT GCCGCGGCTT TGACGACTAA ATACTATTGT TGGTCGCAGG

XmaI
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SmaI  
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AvaI
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Bsu36I  
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251 CTCAGGCGTG CCGGATCGTT TTAGCGGATC CAAAAGCGGC ACCAGCGCGA  
GAGTCCGCAC GGCCTAGCAA AATCGCCTAG GTTTCGCCG TGGTCGCGCT

BpuAI  
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BbsI
~~~~~

301 GCCTTGCAT TACGGGCCTG CAAAGCGAAG ACGAAGCGGA TTATTATTGC  
CGGAACGCTA ATGCCCGGAC GTTTCGCTTC TGCTTCGCT ATAATAACG

Bsu36I  
~~~~~

351 CAGAGCTATG ACATGCCTCA GGCTGTGTTT GGCGGCGGCA CGAAGTTAA
GTCTCGATAC TGTACGGAGT CCGACACAAA CCGCCGCCGT GCTTCAAATT

MscI
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DraIII  
~~~~~

401 CCGTTCTTGG CCAGCCGAAA GCCGCACCGA GTGTGACGCT GTTTCCGCCG
GGCAAGAACCG GGTGGCTTT CGGCCTGGCT CACACTGCGA CAAAGGCGGC

451 AGCAGCGAAG AATTGCAGGC GAACAAAGCG ACCCTGGTGT GCCTGATTAG
TCGTCGCTTC TTAACGTCCG CTTGTTCGC TGGGACCACA CGGACTAATC

501 CGACTTTAT CGGGAGCCG TGACAGTGGC CTGGAAGGCA GATAGCAGCC

41/49

GCTGAAAATA GGCCCTCGGC ACTGTCACCG GACCTTCCGT CTATCGTCGG
 551 CCGTCAAGGC GGGAGTGGAG ACCACCACAC CCTCCAAACA AAGCAACAAC
 GGCAGTTCCG CCCTCACCTC TGGTGGTGTG GGAGGTTTGT TTCGTTGTTG
 601 AAGTACGCGG CCAGCAGCTA TCTGAGCCTG ACGCCTGAGC AGTGGAAAGTC
 TTCAATGCGCC GGTCTCGAT AGACTCGGAC TGCGGACTCG TCACCTTCAG
 651 CCACAGAACG TACAGCTGCC AGGTACGCA TGAGGGGAGC ACCGTGGAAA
 GGTGTCTTCG ATGTCGACGG TCCAGTGC GT ACTCCCCTCG TGGCACCTTT

StuI SphI

~~~~~ ~~~~~

701 AAACCGTTGC GCCGACTGAG GCCTGATAAG CATGCGTAGG AGAAAATAAA  
 TTTGGCAACG CGGCTGACTC CGGACTATTG GTACGCATCC TCTTTTATTT  
 751 ATGAAACAAA GCACTATTGC ACTGGCACTC TTACCGTTGC TCTTCACCCC  
 TACTTTGTTT CGTGATAACG TGACCGTGAG AATGGCAACG AGAAGTGGGG

MfeI

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801 TGTTACCAAA GCCCAGGTGC AATTGAAAGA AAGCGGCCCG GCCCTGGTGA
 ACAATGGTTT CGGGTCCACG TTAACCTTCT TTGCGCCGGC CGGGACCAC

BspEI

~~~~~

851 AACCGACCCA AACCTGACC CTGACCTGTA CCTTTTCCGG ATTAGCCTG  
 TTGGCTGGGT TTGGGACTGG GACTGGACAT GGAAAAGGCC TAAATCGGAC  
 901 TCCACGTCTG GCGTGGCGT GGGCTGGATT CGCCAGCCGC CTGGGAAAGC  
 AGGTGCAGAC CGCAACCGCA CCCGACCTAA CGGGTCGGCG GACCCTTCG

XbaI

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AvaI

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951 CCTCGAGTGG CTGGCTCTGA TTGATTGGGA TGATGATAAG TATTATAGCA  
 GGAGCTCACC GACCGAGACT AACTAACCT ACTACTATTC ATAATATCGT

BstBI

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SfuI

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NspV

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1001 CCAGCCTGAA AACCGTCTG ACCATTAGCA AAGATACTTC GAAAAATCAG
 GGTGGACTT TTGCGCAGAC TGGTAATCGT TTCTATGAAG CTTTTTAGTC

1051 GTGGTGCTGA CTATGACCAA CATGGACCCG GTGGATACGG CCACCTATTA
 CACCACGACT GATACTGGTT GTACCTGGGC CACCTATGCC GGTGGATAAT

BssHII

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StyI

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1101 TTGCGCGCGT TCTCCTCGTT ATCGTGGTGC TTTTGATTAT TGGGGCCAAG
 AACCGCGCGCA AGAGGAGCAA TAGCACCACG AAAACTAATA ACCCCGGTTC

BpuI

StyI

CellII

1151 GCACCCCTGGT GACGGTTAGC TCAGCGTCGA CCAAAGGTCC AAGCGTGTGTT
 CGTGGGACCA CTGCCAATCG AGTCGAGCT GGTTTCCAGG TTCGCACAAA

1201 CCGCTGGCTC CGAGCAGCAA AAGCACCAAGC GGCAGCACGG CTGCCCTGGG
 GGCGACCGAG GCTCGTCGTT TTCGTGGTCG CCGCCGTGCC GACGGGACCC

1251 CTGCCTGGTT AAAGATTATT TCCCAGGAACC AGTCACCGTG AGCTGGAACA
 GACGGACCAA TTTCTAATAA AGGGCCTTGG TCAGTGGCAC TCGACCTTGT

1301 GCGGGGCGCT GACCAGCGGC GTGCATACCT TTCCGGCGGT GCTGCAAAGC
 CGCCCCGCGA CTGGTCGCCG CACGTATGGA AAGGCCGCCA CGACGTTTCG

1351 AGCGGCCTGT ATAGCCTGAG CAGCGTTGTG ACCGTGCCGA GCAGCAGCTT
 TCGCCGGACA TATCGGACTC GTCGAACAC TGGCACGGCT CGTCGTCGAA

1401 AGGCACTCAG ACCTATATTG GCAACGTGAA CCATAAACCG AGCAACACCA
 TCCGTGAGTC TGGATATAAA CGTTGCACTT GGTATTGGC TCGTTGTGGT

EcoRI

1451 AAGTGGATAA AAAAGTGGAA CCGAAAAGCG AATTGGGGGG AGGGAGCGGG
 TTCACCTATT TTTTACACCTT GGCTTTCGC TTAAGCCCCC TCCCTCGCCC

1501 AGCGGTGATT TTGATTATGA AAAGATGGCA AACGCTAATA AGGGGGCTAT
 TCGCCACTAA AACTAATACT TTTCTACCGT TTGCGATTAT TCCCCCGATA
 gIIIseq9 100.0%

=====

1551 GACCGAAAAT GCCGATGAAA ACGCGCTACA GTCTGACGCT AAAGGCAAAC
 CTGGCTTTA CGGCTACTTT TGCGCGATGT CAGACTGCGA TTTCCGTTG

ClaI

1601 TTGATTCTGT CGCTACTGAT TACGGTGCTG CTATCGATGG TTTCATTGGT
 AACTAAGACA GCGATGACTA ATGCCACGAC GATAGCTACC AAAGTAACCA

1651 GACGTTTCCG GCCTTGCTAA TGGTAATGGT GCTACTGGTG ATTTTGCTGG
 CTGCAAAGGC CGGAACGATT ACCATTACCA CGATGACCCAC TAAAACGACC

1701 CTCTAATTCC CAAATGGCTC AAGTCGGTGA CGGTGATAAT TCACCTTAA
 GAGATTAAGG GTTTACCGAG TTCAGCCACT GCCACTATTA AGTGGAAATT

1751 TGAATAATTG CCGTCAATAT TTACCTTCCC TCCCTCAATC GGTTGAATGT
 ACTTATTAAA GGCAGTTATA AATGGAAGGG AGGGAGTTAG CCAACTTACA

1801 CGCCCTTTG TCTTGGCGC TGGTAAACCA TATGAATTCTT CTATTGATTG
 GCGGGAAAAC AGAAACCGCG ACCATTGGT ATACTTAAAA GATAACTAAC

1851 TGACAAAATA AACTTATTCC GTGGTGTCTT TGCCTTCTT TTATATGTTG
 ACTGTTTAT TTGAATAAGG CACCACAGAA ACGCAAAGAA AATATACAAAC

1901 CCACCTTTAT GTATGTATTG TCTACGTTTG CTAACATACT GCGTAATAAG
 GGTGGAAATA CATAACATAAA AGATGCAAAC GATTGTATGA CGCATTATTC

HindIII

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1951 GAGTCTTGAT AAGCTTGACC TGTGAAGTGA AAAATGGCGC AGATTGTGCG  
 CTCAGAACTA TTCGAACCTGG ACACTTCACT TTTTACCGCG TCTAACACGC  
 OGIIII3 100.0%

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2001 ACATTTTTT TGTCTGCCGT TTAATGAAAT TGTAAACGTT AATATTTGT  
 TGAAAAAAA ACAGACGGCA AATTACTTTA ACATTTGCAA TTATAAAACA

2051 TAAAATTTCG GTTAAATTTT TGTTAAATCA GCTCATTTT TAACCAATAG  
 ATTTAAGCG CAATTAAAAA ACAATTAGT CGAGTAAAAA ATTGGTTATC

2101 GCCGAAATCG GCAAAATCCC TTATAAATCA AAAGAATAGA CCGAGATAGG  
 CGGCTTAGC CGTTTAGGG AATATTAGT TTTCTTATCT GGCTCTATCC

2151 GTTGAGTGTGTT GTTCCAGTTT GGAACAAGAG TCCACTATTA AAGAACGTGG  
 CAACTCACAA CAAGGTCAA CTTGTTCTC AGGTGATAAT TTCTTGACCC

2201 ACTCCAACGT CAAAGGGCGA AAAACCGTCT ATCAGGGCGA TGGCCCACCA  
 TGAGGTTGCA GTTTCCCGCT TTTTGGCAGA TAGTCCCGCT ACCGGGTGAT

2251 CGAGAACCAT CACCCTAACAT AAGTTTTTG GGGTCGAGGT GCCGTAAGC  
 GCTCTGGTA GTGGGATTAG TTCAAAAAAC CCCAGCTCCA CGGCATTCG

2301 ACTAAATCGG AACCTAAAG GGAGCCCCCG ATTTAGAGCT TGACGGGGAA  
 TGATTTAGCC TTGGGATTTC CCTCGGGGGC TAAATCTCGA ACTGCCCTT

2351 AGCCGGCGAA CGTGGCGAGA AAGGAAGGGA AGAAAGCGAA AGGAGCGGGC  
 TCGGCCGCTT GCACCGCTCT TTCCTCCCT TCTTCGCTT TCCTCGCCCC

2401 GCTAGGGCGC TGGCAAGTGT AGCGGTACG CTGCGCGTAA CCACCACACC  
 CGATCCCGCG ACCGTTACAC TCGCCAGTGC GACGCGCATT GGTGGTGTGG

2451 CGCCCGCTT AATGCGCCGC TACAGGGCGC GTGCTAGCCA TGTGAGCAAA  
 CGGGCGCGAA TTACCGGGCG ATGTCCCGCG CACGATCGGT ACACTCGTTT

2501 AGGCCAGCAA AAGGCCAGGA ACCGTAaaaa GGCGCGTTG CTGGCGTTTT  
 TCCGGTCGTT TTCCGGTCCT TGGCATTTTT CGGGCGCAAC GACCGAAAAA

2551 TCCATAGGCT CCGCCCCCT GACGAGCATC ACAAAATCG ACGCTCAAGT  
 AGGTATCCGA GGCGGGGGGA CTGCTCGTAG TGTTTTAGC TGCGAGTTCA

2601 CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC  
 GTCTCCACCG CTTGGGCTG TCCTGATATT TCTATGGTCC GCAAAGGGGG

2651 TGGAAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT  
 ACCTTCGAGG GAGCACGCGA GAGGACAAGG CTGGGACGGC GAATGGCCTA

2701 ACCTGTCCGC CTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA  
 TGGACAGGCG GAAAGAGGGA AGCCCTTCGC ACCGCGAAAG AGTATCGAGT

2751 CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG  
 GCGACATCCA TAGAGTCAAG CCACATCCAG CAAGCGAGGT TCGACCCGAC

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44/49

2801 TGTGCACGAA CCCCCCGTTC AGTCCGACCG CTGCGCCTTA TCCGGTAACT
 ACACGTGCTT GGGGGGCAAG TCAGGCTGGC GACGCCGAAT AGGCCATTGA

 2851 ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA
 TAGCAGAACT CAGGGTGGGC CATTCTGTGC TGAATAGCGG TGACCGTCGT

 2901 GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA
 CGGTGACCAT TGTCCCTAACATC GTCTCGCTCC ATACATCCGC CACGATGTCT

 2951 GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGA ACAGTATTTG
 CAAGAACTTC ACCACCGGAT TGATGCCGAT GTGATCTTCT TGTCATAAAC

 3001 GTATCTGCGC TCTGCTGTAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC
 CATAGACGCG AGACGACATC GGTCAATGGA AGCCTTTTC TCAACCATCG

 3051 TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTGTTTG
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 3101 CAAGCAGCAG ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTGA
 GTTCGTCGTC TAATGCGCGT CTTTTTTCC TAGAGTTCTT CTAGGAAACT

 3151 TCTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC ACGTTAAGGG
 AGAAAAGATG CCCCAGACTG CGAGTCACCT TGCTTTGAG TGCAATTCCC

 3201 ATTTTGGTCA GATCTAGCAC CAGGCCTTA AGGGCACCAA TAACTGCCTT
 TAAAACCAGT CTAGATCGTG GTCCGCAAAT TCCCGTGGTT ATTGACGGAA

 3251 AAAAAAATTA CGCCCCGCC TGCCACTCAT CGCAGTACTG TTGTAATTCA
 TTTTTTAAT GCGGGGCGGG ACGGTGAGTA GCGTCATGAC AACATTAAGT

 3301 TTAAGCATTG TGCCGACATG GAAGCCATCA CAAACGGCAT GATGAACCTG
 AATTGTAAG ACGGCTGTAC CTTCGGTAGT GTTGCCGTA CTACTTGGAC

 3351 AATCGCCAGC GGCATCAGCA CCTTGTGCC TTGCGTATAA TATTGCCCA
 TTAGCGGTCG CCGTAGTCGT GGAACAGCGG AACGCATATT ATAAACGGGT

 3401 TAGTGAAAAC GGGGGCGAAG AAGTTGTCCA TATTGGCTAC GTTTAAATCA
 ATCACTTTG CCCCCGCTTC TTCAACAGGT ATAACCGATG CAAATTTAGT

 3451 AAACCTGGTGA AACTCACCCA GGGATTGGCT GAGACGAAAA ACATATTCTC
 TTGACCACT TTGAGTGGGT CCCTAACCGA CTCTGCTTT TGTATAAGAG

 3501 AATAAACCCCT TTAGGGAAAT AGGCCAGGTT TTCACCGTAA CACGCCACAT
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 3551 CTTGCGAATA TATGTGTAGA AACTGCCGGA AATCGTCGTG GTATTCACTC
 GAACGCTTAT ATACACATCT TTGACGGCCT TTAGCAGCAC CATAAGTGAG

+1

3601 CAGAGCGATG AAAACGTTTC AGTTTGTCA TGGAAAACGG TGTAACAAGG
 GTCTCGCTAC TTTTGCAAAG TCAAACGAGT ACCTTTGCC ACATTGTTCC

 3651 GTGAACACTA TCCCACATCA CCAGCTCACC GTCTTCATT GCCATACGGA
 CACTTGTGAT AGGGTATAGT GGTCGAGTGG CAGAAAGTAA CGGTATGCC

 3701 ACTCCGGGTG AGCATTCAATC AGGCAGGGCAA GAATGTGAAT AAAGGCCGGA
 TGAGGCCAC TCGTAAGTAG TCCGCCGTT CTTACACTTA TTTCCGGCCT

3751 TAAAACTTGT GCTTATTTTT CTTTACGGTC TTTAAAAAGG CCGTAATATC
ATTTTGAACA CGAATAAAAAA GAAATGCCAG AAATTTTCC GGCATTATAG

3801 CAGCTGAACG GTCTGGTTAT AGGTACATTG AGCAACTGAC TGAAATGCCT
GTCGACTTGC CAGACCAATA TCCATGTAAC TCGTTGACTG ACTTTACGGA

3851 CAAAATGTTC TTTACGATGC CATTGGGATA TATCAACGGT GGTATATCCA
GTTTTACAAG AAATGCTACG GTAACCCTAT ATAGTTGCCA CCATATAGGT

3901 GTGATTTTTT TCTCCATTTC AGCTTCCTTA GCTCCTGAAA ATCTCGATAA
CACTAAAAAA AGAGGTAAAA TCGAAGGAAT CGAGGACTTT TAGAGCTATT

3951 CTCAAAAAAT ACGCCCGGTA GTGATCTTAT TTCATTATGG TGAAAGTTGG
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4001 AACCTCACCC GACGTCTAAT GTGAGTTAGC TCACTCATTA GGCACCCCAG
TTGGAGTGAGG CTGCAGATTA CACTCAATCG AGTGAGTAAT CCGTGGGGTC

4051 GCTTTACACT TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG
CGAAATGTGA AATACGAAGG CCGAGCATAAC AACACACCTT AACACTCGCC

M13 Reverse primer 100.0%

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4101 ATAACAATTTCACACAGGAA ACAGCTATGA CCATGATTAC GAATT
TATTGTTAAA GTGTGTCCTT TGTCGATACT GGTACTAATG CTTAA

Figure 15

MS-GPC-1:

VH

QVQLKESGPALVKPTQTLTLCFTSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARQYGHRRGGFDHWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDFNE
SVFGGGTKLTVLG

MS-GPC-6

VH

EVQLVESGGGLVQPQGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLE
WVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY
YCARGYGRYSPDLWGQGTLTVSS

VL

DIVLTQSPATLSLSPGERATLSCRASQS VSSSYLAWYQQKPGQAPRLLI
YGASSRATGVPARFSGSGSGTDFTLTISLEPEDFAVYYCQQYSNLPF
TFGQGTKVEIKRT

MS-GPC-8

VH

QVQLKESGPALVKPTQTLTLCFTSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARS PRYRGAFDYWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI
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AVFGGGTKLTVLG

MS-GPC-10

VH

QVQLKESGPALVKPTQTLTCTFSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARQLHYRGGFDLWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDLTM
GVFGGGTKLTVLG

MS-GPC-8-6

VH

QVQLKESGPALVKPTQTLTCTFSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPRYRGAFDYWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDYDH
YVFGGGTKLTVLG

MS-GPC-8-10

VH

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WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPTYRGAFDYWGQGTLTVSS
VL
DIVLTQPPSVSGAPGQRVTISCSGSSNIGSNYVSWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDLIRH
VFGGGTKLTVLG

MS-GPC-8-17

VH

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WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPTYRGAFDYWGQGTLTVSS
VL
DIVLTQPPSVSGAPGQRVTISCSGSSNIGSNYVSWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDFSV
YVFGGGTKLTVLG

MS-GPC-8-27

VH

QVQLKESGPALVKPTQTLTLCFSGFSLSTSGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPTYRGAFDYWGQGTLTVSS
VL
DIVLTQPPSVSGAPGQRVTISCSGSSNIGSNYVSWYQQLPGTAPKLLI
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HVFGGGTKLTVLG

MS-GPC-8-6-13

VH

QVQLKESGPALVKPTQTLTCTFSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPTYRGAFDYWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSESNIGANYVTWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDYDH
YVFGGGTKLTVLG

MS-GPC-8-10-57

VH

QVQLKESGPALVKPTQTLTCTFSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPTYRGAFDYWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSESIGNNYVQWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDLIRH
VFGGGTKLTVLG

MS-GPC-8-27-41

VH

QVQLKESGPALVKPTQTLTCTFSGFSLSTSGVGVGWIRQPPGKALE
WLALIDWDDDKEYSTSLKTRLTISKDTSKNQVVLTMTNMDPVDTATYY
CARSPTYRGAFDYWGQGTLTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSESIGNNYVQWYQQLPGTAPKLLI
YDNNQRPSGVPDFSGSKSGTSASLAITGLQSEDEADYYCQSYDMNV
HVFGGGTKLTVLG

INTERNATIONAL SEARCH REPORT

Inte nal application No.
PC1/US01/15625

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7)- :A61K 39/395, 44

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/180.1, 188.1, 188.1, 141.1, 148.1, 144.1, 152.1, 153.1, 155.1, 172.1, 173.1, 174.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CANCERLIT, WEST
search terms antibodies, apoptosis, HLA-DR**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DUEYMES et al. Anti-endothelial cell antibody binding causes apoptosis of endothelial cells. Arthritis & Rheumatism. September 1997, Vol.40, page S103. See abstract.	1 ---
Y	KIM et al. Altered expression of the genes regulating apoptosis in multidrug resistant human myeloid leukemia cell lines overexpressing MDR1 or MRP gene. 1997, Vol.11, pages 945-950. See abstract.	109, 110 ---
X	ISHIZUKA et al. Antitumour activity of murine monoclonal antibody NCC-ST-421 on human cancer cells by inducing apoptosis. Anticancer Research. July-August 1998, Vol.18, pages 2513-2518. See abstract.	1 ---
Y		109-110

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

29 JULY 2001

Date of mailing of the international search report

15 AUG 2001

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US01/15625

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GHAHREMANI et al. Activation of fas ligand/receptor system kills ovarian cancer cell lines by apoptotic mechanism.	1 ---
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Y	Gynecological Oncology. August 1998, Vol.70, pages 275-281. See abstract.	109, 110
X	HENSEL et al. Characterization of glycosylphosphatidylinositol-linked molecule CD55decay-accelerating factor as the receptor for antibody SC-1-induced apoptosis. Cancer Research. 15 October 1999, Vol.59, pages 5299-5306. See abstract.	1 ---
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Y		109, 110
X	NAKAMURA et al. Apoptosis induction of the human lung cancer cell line in multicellular heterospheres with human	1 ---
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Y	antigangliosides GM2 monoclonal antibody. Cancer Research. 15 October 1999, Vol.59, pages 5323-5330. See abstract.	109, 110
X	WALLEN-OHMAN et al. Antibody-induced apoptosis in a human leukemia cell line is energy dependent. Cancer Letters. 10 December 1993, Vol.75, pages 103-109. See abstract.	1 ---
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Y		109, 110
X	MYSLER et al. The apoptosis-1/Fas protein in human systemic lupus erythematosus. Journal of Clinical Investigation. March 1994, Vol.93, pages 1029-1034. See abstract.	1 ---
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Y		109, 110
X	ACKERMAN et al. Induction of apoptotic or lytic cell death in an ovarian adenocarcinoma cell line by antibodies generated against a synthetic N-terminal extracellular domain gonadotropin-releasing hormone receptor peptide. Cancer Letters. 30 June 1994, Vol.81, pages 177-184. See abstract.	1 ---
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Y		109, 110
X	ERAY et al. Cross-linking of surface IgG induces apoptosis in bcl-2 expressing human follicular lymphoma line of mature B cell phenotype. International Immunology. December 1994, Vol.6, pages 1817-1827. See abstract.	1 ---
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Y		109, 110
X	NAKAMURA et al. Apoptosis in human hepatoma cell line induced by 4,5-dihydro geranylgeranoic acid via down-regulation of transforming growth factor-alpha. Biochemical and Biophysical Research Communications. 06 February 1996, Vol.219, pages 100-104. See abstract.	1 ---
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Y		109, 110
X	VOLLMERS et al. Apoptosis of stomach carcinoma cells induced by a human monoclonal antibody. Cancer. 15 August 1995, Vol.76, pages 550-558. See abstract.	1 ---
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Y		109, 110
X	HATA et al. Fas/Apo-1 (CD95)-mediated and CD95-independent apoptosis of malignant plasma cells. Leukemia and Lymphoma. December 1996, Vol.24, pages 35-42. See abstract.	1

INTERNATIONAL SEARCH REPORT

Inte
nal application No.
PC17 US01/15625

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIGASHIGAWA et al. FK506 inhibits anti-IgM antibody-induced apoptosis and 17 kD endonuclease activity in the human B-cell line, MBC-1, established from Burkitt's lymphoma. British Journal of Haematology, December 1997, Vol.99, pages 908-913. See abstract.	1 ---
X	MASUDA et al. Dual action of CD30 antigen: anti-CD30 antibody induced apoptosis and interleukin-8 secretion in Ki-1 lymphoma cells. International Journal of Hematology, April 1998, Vol.67, pages 257-265. See abstract.	1 ---
X	HAYAKAWA et al. A short peptide derived from the antisense homology box of Fas ligand induces apoptosis in anti-Fas antibody-insensitive huamn ovarain cancer cells. Apoptosis. February 2000, Vol.5, pages 37-41. See entire document.	1 ---
X	VIDOVIC et al. Selective apoptosis of neoplastic cells by the HLA-DR-specific monoclonal antibody. Cancer Letters. 19 June 1998, Vol.128, pages 127-135. See entire document.	1-5, 7-10, 18, 19, 21 ----- 6, 14, 15, 38, 66, 68, 69, 70, 109- 114
X	LEE et al. HLA-DR-Triggered Inhibititon of Hemopoiesis Involves Fas/Fas Ligand Interactions and Is Prevented by c-kit Ligand. Journal of Immunology. 01 October 1997, Vol.159, pages 3211-3219. See entire document.	1-5, 7-10 ----- 6, 14, 15, 38, 69 109- 114
X	LEE et al. HLA-DR-Mediated Signals for Hematopoiesis and Induction of Apoptosis Involve But Are Not Limited to a Nitric Oxide Pathway. Blood. 01 July 1997, Vol.90, pages 217-225. See entire document.	1-5, 7-10 ----- 6, 14, 15, 38, 69 109-114
X	McDEVITT et al. Monoclonal anti-Ia antibody therapy in animal models of autoimmune disease. Ciba Foundation Symposium. 1987, Vol.129, pages 184-193. See entire document.	1-5, 7-10, 66, 68, 70 ----- 6, 14, 15, 38, 69
Y	HARRISON et al. Screening of Phage Antibody Libraries. Methods in Enzymology. 1996, Vol.267, pages 83-109. See entire document.	6, 69
Y	ROOS et al. Establishment and characterization of a human EBV-negative B cell line. Leukemia Research. 1982, Vol.6, pages 685-693. See abstract.	14, 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/15625

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TOSI et al. Immunochemical definition of the new dr specificity 8WDRw13. Immunological Communications. 1981, Vol.10, pages 275-292. See abstract.	14, 15

INTERNATIONAL SEARCH REPORT

Interr [✉] al application No.
PCT/US01/15625

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/180.1, 183.1, 188.1, 141.1, 148.1, 144.1, 152.1, 153.1, 155.1, 172.1, 173.1, 174.1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/15625

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: 11-18,24-37,39-65,71-108 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.